COMPARATIVE CHEMICAL AND BIOLOGICAL STUDIES ON THE FRACTIONATION OF DEOXYRIBONUCLEIC ACIDS

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INTRODUCTION

Chemical and Biological Heterogeneity of Deoxyribonucleate

Near the end of the nineteenth century, Miescher isolated an acid-insoluble phosphorus - rich material from salmon sperm, and from the nuclei of pus cells and other tissues. He termed this material "nuclein" (1). Later workers showed that nuclein was, in fact, a mixture of at least two substances, one containing the sugar, ribose, and the other, the sugar, deoxyribose. This latter type of nuclein, which was confined almost exclusively to cell nuclei, became known as deoxyribonucleic acid.

Much of the foundation of our present knowledge of the chemical structure of nucleic acids was established by P. A. Levene (2). More recently, through the development and use of quantitative semi-micro techniques for the analysis and estimation of purines and pyrimidines, Chargaff and his colleagues showed that specimens of deoxyribonucleate from different biological sources frequently differ in their nitrogenous base composition (3). These workers concluded that the nuclei of different species may contain different molecules of deoxyribonucleate. In agreement with this chemical evidence of species-specificity, is the growing body of biological evidence which suggests that genetic information may be embodied in the composition and structure of deoxyribonucleate. Moreover, specimens of deoxyribonucleate from individual sources have recently been found to consist of populations of closely related but chemically distinct molecules. This was first shown by Chargaff, Crampton, and Lipshitz, who extracted fractions of the nucleate having decreasing contents of guanine and cytosine from histone-chloroform gels by means of aqueous solutions of increasing salt concentration (4, 5). Similar findings were obtained by other workers whose techniques also depended upon the specific, fractional dissociation of deoxyribonucleate from cationic adsorbents to which it had been bound by electrostatic linkages (6, 7, 8). However, the elution of deoxyribonucleate from columns of cellulose linked to triethanolamine residues by reaction with epichlorohydrin (ECTEOLA-cellulose) provides successive fractions of essentially unchanging composition, but of gradually increasing average sedimentation constant (9). Thus, another important aspect of the molecular heterogeneity of deoxyribonucleate arises from variation with respect to sedimentation properties (9, 10, 11). Recent studies suggest that molecules in a single specimen of deoxyribonucleate are also heterogeneous with respect to their apparent density when analyzed by centrifugation in solutions of CsCl (12). Such studies indicate that apparent density is related directly to base composition. Evidence has also been presented for a relationship between base composition and thermal stability of deoxyribonucleate (13).

With few exceptions, the amount of deoxyribonucleate per nucleus in cells of a given species is a multiple of the haploid amount, and is characteristic of the species (14). If the two daughter cells arising from division contain the same amount of deoxyribonucleate as the parent cell, division must be preceded or accompanied by a net synthesis of deoxyribonucleate. At present, little is known regarding those factors which initiate and govern the synthesis of the various molecules of deoxyribonucleate present in mammalian cells or of the mechanisms which control the constancy of their amounts per nucleus. That the net synthesis of deoxyribonucleate is confined to interphase in cells from several mammalian tissues, was shown in 1950 by means of cytochemical techniques (15). This finding was confirmed by radioautographic evidence (16), and has recently been extended to Ehrlich ascites tumor cells (17). As has been shown by several groups of workers (18, 19), the deoxyribonucleate of mammalian cells exhibits extreme metabolic stability, suggesting that it is not degraded during the life of the cell, or that, if degraded, its substance is efficiently reutilized.

The work to be described below stems from the paradoxical finding that deoxyribonucleate, despite its polyanionic character, is bound by columns of the magnesium form of IRC-50, a polycarboxylic acid resin. Elution of the deoxyribonucleate yields successive fractions of gradually changing composition (20). In the present studies, attempts were made 1) to investigate the influence of various factors on the interaction of deoxyribonucleate from calf thymus with Mg IRC-50, 2) to determine the usefulness of this system for the chromatographic fractionation of deoxyribonucleate from rat liver, Pneumococcus, and Ehrlich ascites tumor cells and 3) to compare the initial rates of in vivo incorporation of radioactive precursors into chromatographically different fractions of deoxyribonucleate. The latter studies of incorporation were undertaken with the hope of obtaining information regarding metabolic properties of different molecules of deoxyribonucleate.

EXPERIMENTAL PROCEDURES

Isolation and Purification of Deoxyribonucleates

Deoxyribonucleates from calf thymus and rat liver

Details of the isolation of the specimens from calf thymus and rat liver have been prepared for publication.[1] The specimens of deoxyribonucleate from the thymus of calf #30 were isolated from a nucleohistone which had been extracted by 0.0004 M $NaHCO_3$. Preparations 30A and 30B were obtained by slightly different procedures, but were indistinguishable with respect to all properties which were examined. The specimen from calf #32 thymus was obtained by use of the procedure of Crampton *et al.*[1] which depends on the insolubility of deoxyribonucleoproteins in a solution of 6 M urea containing 1% NaCl and 0.1% merthiolate. Nucleoproteins of the ribose type remain soluble in this medium, the use of which has provided specimens of deoxyribonucleate containing little or no uracil. Deoxyribonucleate was prepared from rat #11 liver using this same method. All of these preparations were deproteinized by two treatments with Duponol by use of the procedure of Kay *et al.* (21).

Deoxyribonucleate from Ehrlich ascites tumor cells

The line of Ehrlich ascites tumor cells[2] was maintained by weekly transfers of approximately 2 x 10^6 cells to groups of hybrid Swiss white mice, fifty to sixty days of age. Cells for inoculation were aspirated by use of sterile, large bore, transfer pipettes from the peritoneal cavities of mice which had been killed by cervical dislocation. About two to four minutes were required to collect the cells which were then centrifuged briefly at room temperature. The packed cells were then suspended in twenty volumes of sterile saline solution. Appropriate aliquots of such suspensions, which contained about 2 x 10^7 cells per ml, were used for inoculation.

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[1] C. F. Crampton, B. Greenfield, J. Adair, and F. R. Frankel, manuscript in preparation.

[2] We are indebted to Dr. C. Heidelberger for supplying the Ehrlich ascites tumor cells used in these studies.

Specimens of deoxyribonucleate designated EA #1 to #17 were isolated from cells donated by the respective groups of uniform mice. The mice of each group received 2 x 10^6 to 20 x 10^6 cells from a single inoculum five to six days prior to sacrifice. When deoxyribonucleate was to be extracted, the cells were collected in the manner described above, except that the peritoneal cavities of the mice were frequently rinsed several times with cold saline in order to increase the yield of cells. Moreover, the cells were always placed immediately in a tube immersed in an ice bath. In studies of the uptake of labeled compounds, the stated intervals of incorporation correspond to the average time which elapsed between injecting the radioactive precursor and chilling the cells. The chilled cells were promptly centrifuged at 4° C, and the sediment remaining after decanting the supernatant fluid was immediately frozen in a mixture of dry ice and ethanol. The pellets were occasionally stored at -20° C for twenty-four hours before being processed further.

In order to extract deoxyribonucleate from the packed cells, the pellets were permitted to thaw partially in the cold room (at 4°-6° C, at which temperature all subsequent procedures were performed). The packed cells (2 to 5 ml) were transferred with five to ten volumes (with respect to the volume of packed cells) of the urea medium to the semi-micro cup of a high speed mixer equipped with cutting blades. The mixer was then operated at full speed for two minutes. In the case of EA #1 and EA #4, the initial homogenates were prepared by use of a Potter-Elvehjem tissue grinder equipped with a plastic pestle. After the addition of two drops of octyl alcohol, in order to collapse the foam, the homogenate was transferred to a plastic centrifuge tube, and the insoluble deoxyribonucleohistone and other cell debris were sedimented by centrifugation at 20,000 x g for ten minutes. The sediment was redispersed completely in five to ten volumes of fresh urea medium with the aid of a mechanically driven pestle which had been machined to fit the centrifuge tube tightly. The suspension was centrifuged as before, and the resuspension and sedimentation were repeated using fresh urea medium. The sediment was then washed two times in the same manner with 0.15 M NaCl. The sediments at this point for preparations EA #1, #4, #5, #6, and #7 were dispersed in 4 ml of distilled water and made about 1 M in NaCl by the addition of 5 ml of 2 M NaCl. In order to reduce the viscosity, 1 to 15 ml of 1 M NaCl were added to dilute the suspensions, which were then stirred for eleven to fifteen hours in order to effect dissociation and solubilization of the histone and deoxyribonucleate. The suspensions in 1 M NaCl were then centrifuged for thirty to sixty minutes at 20,000 x g in order to remove insoluble constituents. Before extraction with 1 M NaCl, the sediment of EA #9 was first extracted with nine volumes, and then with one volume, of 0.2 M $Ba(OAc)_2$ in order to dissociate histone Fraction A (22). The sediments of EA #15, #16, and #17 were extracted with five volumes of 0.2 M $Ba(OAc)_2$ before being dispersed in four volumes of 2 M NaCl. The suspensions in 2 M NaCl were stirred one hour, centrifuged at 20,000 x g for fifteen minutes, and the sediments were re-extracted with 2 M NaCl. The combined, clear to slightly turbid, 1 M or 2 M NaCl supernatant fluids from each experiment were transferred to a 125 ml Erlenmeyer flask which was shaken vigorously after the addition of one volume of 95% ethanol. The fibers of precipitated deoxyribonucleate were washed once with 70% ethanol.

For further deproteinization, the precipitated deoxyribonucleate was suspended in 5 to 10 ml of distilled water, and an equal volume of 0.2 M NaCl, 0.05 M Na citrate was added. Duponol was added to a concentration of 0.45% and the solution was brought to room temperature and stirred for three hours. Solid NaCl was added to a concentration of 1 M, and permitted to dissolve completely, after which the solution was centrifuged at 20,000 x g for fifteen minutes at 4° C. Deoxyribonucleate was precipitated from the supernatant fluid by the addition of an equal volume of 95% ethanol, collected, and washed with 70% ethanol. After stirring the nucleate with 8 to 20 ml of distilled water for one to twelve hours at 4° C, a second Duponol treatment was performed as described above for two hours (EA #1, #4, #5, #6, and #7) or one hour (EA #9, #15, #16, and #17). After the final precipitation by ethanol, the deoxyribonucleate was washed three times with 70% ethanol, three times with 95% ethanol, and dried overnight at room temperature in an evacuated desiccator over anhydrous $CaCl_2$. The product was stored at -20° C.

Table I lists the yields and several properties of some of the specimens of deoxyribonucleate used in these studies. The yields of other specimens from Ehrlich ascites tumor cells, per ml of packed cells, were as follows: EA #1, 4 mg; EA #7, 7 mg; EA #9, 3 mg; EA #15, 7 mg; EA #16, 7 mg; EA #17, 6 mg.

Chromatography of Deoxyribonucleate by Use of Mg IRC-50

Amberlite IRC-50, XE-64 (CG-50, Type 2, presently supplied by Fisher Scientific Co.), was obtained in the hydrogen form as the "through 200" mesh material; it was prepared for chromatography by first converting the resin to the sodium form by adjusting to pH 8 or 9 a 6-liter suspension of the resin in distilled water by the addition of pellets of NaOH. The finest resin particles were removed by repeated decantations of the thoroughly stirred 6-liter aqueous suspension after settling periods of about thirty minutes. After about ten decantations, the settled resin was transferred in small portions either to a 200 mesh or a 325 mesh sieve and screened to yield "through 200" or "through 325" material (23). The material passing through the sieves was collected and slowly washed under gravity on a Buchner funnel with successive 2 to 4 liter portions of 4 N NaOH, water, 4 N HCl, water, 4 N NaOH, water, 4 N HCl, water, acetone, and water. The resin was converted to the magnesium salt by slowly passing 1 M $Mg(OAc)_2$ through the resin bed until the pH of the effluent rose to that of the influent,

TABLE I

Properties of some specimens of deoxyribonucleate

Details of the methods used for the isolation and characterization of these specimens are given in the experimental section.

Source of specimen	Gross yield (mg/g or mg/ml)[b]	Loss of weight on drying[c]	Per cent[a]			$\epsilon(P)_{260}$[d] 0.15 M NaCl
				Protein		
			Phosphorus	Biuret	Ninhydrin	
Calf #30A thymus	19	14.1	8.56	1.2	3.6	6930
Calf #32 thymus	24	13.9	8.28	2.9	4.5	6830
Rat #11 liver	1	14.0	7.23	18	---	7040
Ehrlich ascites tumor cells						
EA #4	2	17.8	8.72	1.2	2.7	6430
EA #5	5	18.6	8.94	3.0	2.3	6850
EA #6	4	17.0	9.11	1.6	4.4	6450

[a] As, per cent of the weight after drying.

[b] The figures refer to actual recoveries per g of tissue or per ml of packed tumor cells.

[c] As, per cent of weight before drying.

[d] Atomic extinction coefficient with respect to phosphorus, at 260 mμ.

about pH 7 or 8. The preparation of the resin was completed by washing with 0.05 M $Mg(OAc)_2$, pH 7 to 8. At this point, one-to-one slurries of the resin in 0.05 M $Mg(OAc)_2$ were prepared and used to pour successive 10 to 15 cm segments of analytical columns (0.9 cm diameter) or preparative columns (2 cm diameter), in the manner described by Moore and Stein (23). Unless otherwise noted, the columns were operated in condenser jackets through which water of $30^\circ \pm 0.2^\circ$ C was circulated. Effluent from the analytical columns was collected at a rate of about 20 ml per hour, while the preparative columns were operated at a rate of flow of about 50 ml per hour. To promote the regular flow of drops of uniform size past the light beam of the drop counter and into the collection tubes, a roll of silver gauze was inserted into the tip of the column (24). The gauze made contact with the fritted glass disc of the chromatograph tube and terminated in a point.

The zinc salt of IRC-50 was prepared by substituting solutions of $Zn(OAc)_2$ of pH 6 for the $Mg(OAc)_2$ solutions in the above procedure. Carboxymethyl-cellulose, with an exchange capacity of 0.39 meq per g (Lot No. 59-1, supplied by Bio-Rad Laboratories) was obtained in the hydrogen form, and was converted to the magnesium form as described above. The sulfonated polystyrene resins (Dowex 50-X2 and Amberlite IR-120, XE-69) were prepared as described previously[3] (25) and converted from the hydrogen forms to the magnesium or zinc forms as for IRC-50.

The sample to be chromatographed contained, in most cases, about 1 mg of deoxyribonucleate per ml of the initial buffer, and was prepared by swirling the nucleate in distilled water at 4° C until the specimen was dissolved. This required up to ten hours. Sufficient 1 M salt solution was then added to yield a final concentration of the starting buffer of 0.05 M. Stirring was continued for about two hours, whereupon the solution was permitted to warm to room temperature, and after the removal of aliquots for the purpose of estimating the total absorbancy, the sample was added carefully to the column. The deoxyribonucleate was eluted by eluents of continuously increasing concentration or in a stepwise manner. Eluents of gradually changing composition were produced by use of a device constructed from a flat bottomed flask which served as the mixing chamber and contained the solutions of lowest concentration, and the upper ground glass joint of a wash bottle through which was introduced from a separatory funnel, the solutions of highest concentration. During operation of the device, the solution in the mixing chamber was stirred by a magnetic bar and the eluent was continuously removed through the delivery tube of the wash bottle. Most of the analytical chromatograms were performed by use of a 500 ml mixing chamber containing 0.05 M $Mg(OAc)_2$, into which flowed 0.4 M $Mg(OAc)_2$. Exploratory experiments indicated that a mixing chamber of this size gave satisfactory results. Smaller mixing chambers gave too few fractions to cut conveniently, while larger mixing chambers gave peaks which were impractically broad. For preparative columns, the volume of the mixing chamber was scaled up by a factor of six, although the ratio of the cross-sectional areas of the two columns was 4.9. The effluent from analytical columns was collected in fractions of 2 to 5 ml while that from preparative columns was collected in fractions of 5 to 20 ml.

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[3] C. F. Crampton, F. R. Frankel, A. M. Benson, and A. E. Wade, submitted for publication.

The absorbancy of these fractions was determined at various wave lengths in the Beckman DU spectrophotometer, using distilled water as a blank. The light path of the quartz absorption cells was 1 cm. In an effort to eliminate the contribution of ultraviolet absorbing constituents in the effluent other than deoxyribonucleate, use was made of the difference between the 260 mμ and 290 mμ absorbancies. Amounts of deoxyribonucleate, expressed as "absorbancy units," were calculated by multiplying the 260 mμ minus 290 mμ absorbancy difference by the total volume of the solution. The observation that one mg of deoxyribonucleate per ml of solution gives an absorbancy difference of 12.3 was used to convert absorbancy units to mg of deoxyribonucleate. Column fractions were stored no longer than forty-eight hours at 4° before further treatment.

To isolate deoxyribonucleate from the column effluent, fractions were combined where appropriate, made 1 M with respect to NaCl by the addition of solid salt, and the nucleate precipitated by the addition of two to three volumes of cold 95% ethanol.[4] The precipitates were washed three times with 70% ethanol and three times with 95% ethanol. The product was dried in an evacuated desiccator over $CaCl_2$ and stored at -20° C. Before precipitating the deoxyribonucleate from pooled samples which contained only small amounts of material, the fractions were first concentrated by use of a rotary evaporator, the bath temperature of which was kept less than 36° C. In some instances, such concentrated solutions were dialyzed at 4° C to equilibrium against distilled water so that the final concentration of $Mg(OAc)_2$ was about 0.5 M.

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[4] To facilitate the isolation of small amounts of pneumococcal nucleate with transforming activity, a known quantity of a solution of deoxyribonucleate from calf thymus was added as "carrier" prior to precipitation with ethanol. This procedure yielded difficultly interpretable data with respect to the transformation assays. An alternative recovery procedure, subsequently developed by Dr. R. D. Hotchkiss, is based on the co-precipitation of the nucleate with magnesium phosphate, and the subsequent solution of the deoxyribonucleate by use of ethylenediaminetetraacetate.

Analysis and Quantitative Estimation of Purines and Pyrimidines in Hydrolysates of Deoxyribonucleates

Use of columns of Dowex 50-X4

In order to determine the base composition of specimens of deoxyribonucleate as well as of fractions of deoxyribonucleate isolated after chromatography by use of Mg IRC-50, the procedures described by Crampton *et al.*[3] were used. A specimen of the material to be analyzed was hydrolyzed with 99% formic acid in an evacuated sealed tube for thirty minutes at 175° C. Aliquots of the hydrolysate, after removal of formic acid in an evacuated desiccator and solution of the residue in 1 N sulfuric acid, were taken for phosphorus determination (26) and chromatographic analysis. The analyses were performed by eluting the bases from columns of Dowex 50-X4 (60 x 0.9 cm) using an eluent of gradually increasing concentration of ammonium formate of pH 4. The column effluent was collected in 3 ml fractions, and the absorbancy of each fraction was determined in the ultraviolet spectrophotometer, using distilled water as a blank. A typical analysis is shown in Fig. 1*B*. To estimate the amount of material in each position of the chromatogram, factors obtained by chromatographing mixtures of standard bases under

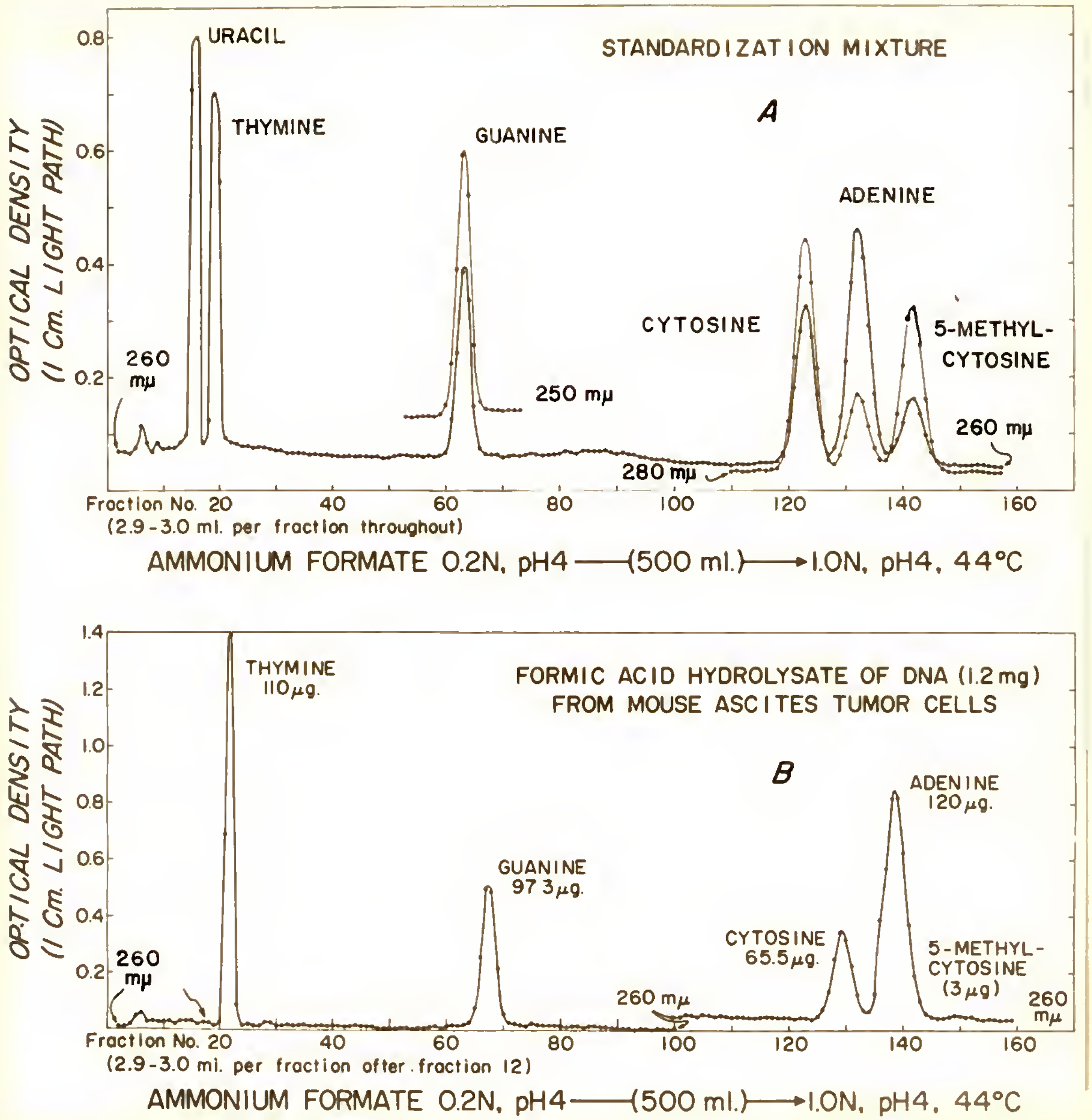

Fig. 1. Chromatography of purines and pyrimidines by use of 60 x 0.9 cm columns of Dowex 50-X4, "through 200." (A) Standardization mixture of approximately 50 μg of each base. (B) Formic acid hydrolysate of 1.2 mg of deoxyribonucleate from Ehrlich ascites tumor cells.

the same conditions (Fig. 1A) were used to convert units of absorbancy to μmoles of base.

Paper chromatography

Frequently, an alternative method of chromatography was used for the isolation of bases for measurements of specific radioactivity. The residue resulting from the formic acid hydrolysis of about 0.6 to 1.0 mg of deoxyribonucleate was dissolved in two drops of 1 N HCl and spotted in duplicate at the origin of a 19.5 cm x 40 cm sheet of Whatman No. 4 filter paper, leaving three lanes to serve as blanks. This was hung in a sealed chromatography jar, and developed by descending chromatography for twenty-five to thirty-five hours at room temperature (23°-26° C) with isopropanol which was 1.96 N with respect to HCl (27). The chromatogram was dried in a stream of warm air. The bases were located by viewing the chromatogram under an ultraviolet lamp,[5] and were cut out, along with the adjacent blank spots, using a pattern to assure elution of bases and blanks from equal areas of paper. The paper segments were rolled into cylinders and deposited in 10 x 75 mm test tubes. To each test tube was delivered 2 ml of eluting solvent using a single calibrated volumetric pipette. Guanine and its blank were eluted with 1 N HCl, while the other bases were eluted with

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[5] When dried and viewed under ultraviolet light, chromatograms of bases which had been recovered from the effluent of columns of Dowex 50 showed a single dark spot at the position of the authentic base, as well as a bright yellow fluorescent spot which moved faster than thymine. This fluorescent spot was seen whenever any base, isolated from Dowex 50 effluent, was chromatographed on paper, but not when the bases were added to the paper from stock solutions of the bases, or directly from hydrolysates. Evidently, the fluorescent material is derived from the sulfonated polystyrene resin.

0.1 N HCl. Thymine containing tritium was eluted with water. The tubes were tightly covered with parafilm and allowed to stand twelve hours at room temperature. At the end of this period, the absorbancy of a portion of the eluate was determined in the ultraviolet spectrophotometer, and aliquots of the remaining eluate were plated for determination of specific radioactivity.

Radioisotope Counting Techniques

Carbon-14 labeled compounds

Bases containing carbon-14 were isolated from the pooled effluent of columns of Dowex 50 by lyophilization of the ammonium formate solution in 250 ml flasks with the aid of infra-red lamps directed at the frozen solution. After the first lyophilization, the residue was dissolved in 5 ml of water and relyophilized. The final residue was taken up in 1 N HCl (guanine) or 0.1 N HCl (thymine, cytosine, adenine), diluted to 2 ml with the same solvent, and an aliquot taken for determination of absorbancy. A portion of the remaining solution was plated for determination of radioactivity. Bases labeled with carbon-14 and isolated by paper chromatography were prepared as described in the previous section.

Compounds dissolved in 0.1 N HCl could not be plated directly on metal. They were therefore applied to aluminum planchets onto which 1.8 cm diameter glass cover slips were secured with the aid of a small amount of silicone grease. Four equal aliquots containing about 5 μg of the base were successively spread on the glass cover slip uniformly covering the glass area, evaporated to dryness under an infra-red lamp, and counted by use of a flow-window device. Counting was performed at 130 volts above the starting voltage. A minimum of 3000 counts was accumulated for each sample. All counting rates were corrected for background. A straight line could in most cases be drawn to connect the points when counts per minute was plotted against total volume plated, indicating that counting was performed under conditions of infinitesimal thickness. The slope of the line which joined the counting rates of the four successive aliquots was divided by the μmoles of base in the sample, determined spectrophotometrically, to obtain the specific activity of the sample.

Tritium labeled compounds

Thymine containing tritium was dissolved in water so that it could be plated and counted on steel planchets. For this purpose, two equal aliquots of 50 μl containing about 0.6 μg of base were successively spread uniformly on a planchet previously washed thoroughly with detergent, pickled by brief immersion in 6 N HCl, washed finally with water and acetone, dried with a stream of warm air, and rimmed with silicone grease. The planchets, with an effective diameter of 2.7 cm, were dried under an infra-red lamp on a rotating platform in a stream of air, and counted in a windowless flow counter operated with 99.05% helium and 0.95% isobutane. Counting was performed 175 volts above the starting voltage. The specific activities of the two aliquots were extrapolated to zero mass to obtain the specific activity of the sample in the absence of self absorption.

RESULTS AND DISCUSSION

Studies of the Interaction Between Mg IRC-50 and Deoxyribonucleate

Specificity of the interaction

Under certain conditions, deoxyribonucleate is adsorbed by the magnesium form of the polycarboxylate resin, Amberlite IRC-50. Fig. 2A illustrates an experiment in which 1 mg of deoxyribonucleate from calf #30B thymus was dissolved in 0.05 M $Mg(OAc)_2$, pH 7.6 and added to a column of Mg IRC-50 which had been equilibrated with an eluent of the same composition as the solvent. Virtually all of the nucleate was bound. The binding was reversible in that about 90% of the nucleate was eluted by applying an eluent of gradually increasing $Mg(OAc)_2$ concentration. The eluent was produced by adding 0.5 M $Mg(OAc)_2$ to a 50 ml mixing chamber initially filled with 0.05 M $Mg(OAc)_2$. As shown in Fig. 2B, a slower rate of increase of the salt concentration in the eluent (achieved by use of a 125 ml mixing chamber) provided a broader elution pattern with the same specimen. The concentrations of $Mg(OAc)_2$ sufficient to elute most of the molecules of deoxyribonucleate were determined by stepwise application of solutions of successively higher molarity. As shown in Fig. 3, obtained with the specimen from calf #32 thymus, elution occurred over the relatively narrow range of 0.20 M to 0.25 M $Mg(OAc)_2$.

In order to determine whether the elution of successive fractions is specific, the composition was examined of three successive

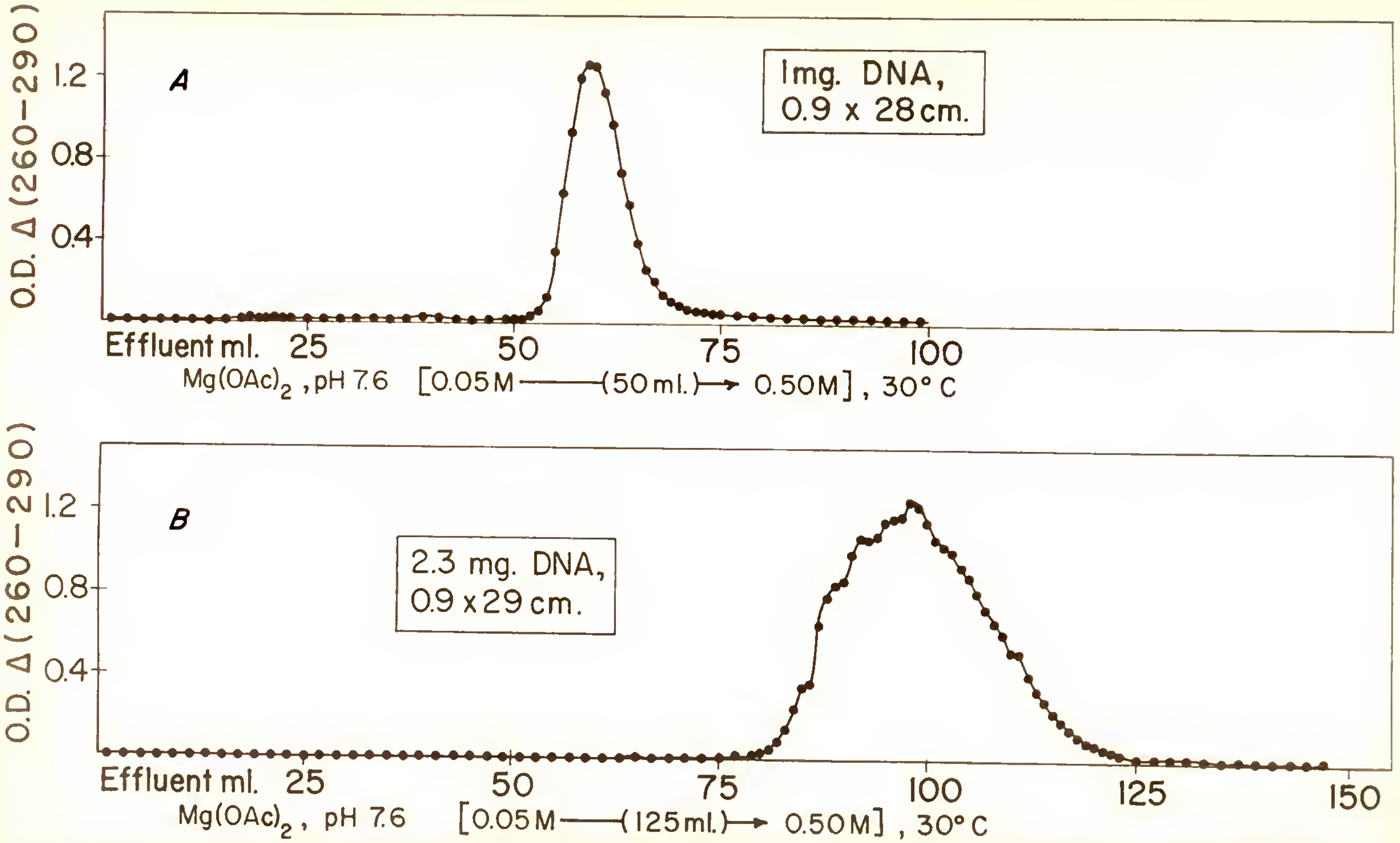

Fig. 2. Chromatography of deoxyribonucleate by use of (A) a 50 ml mixing chamter or (B) a 125 ml mixing chamber with columns of Mg IRC-50, "through 200." The 260 mμ minus 290 mμ absorbancy difference, [O.D. Δ (260-290)], determined as described in the experimental section, is plotted against effluent ml. The sample of deoxyribonucleate for both experiments was preparation 30B from calf thymus.

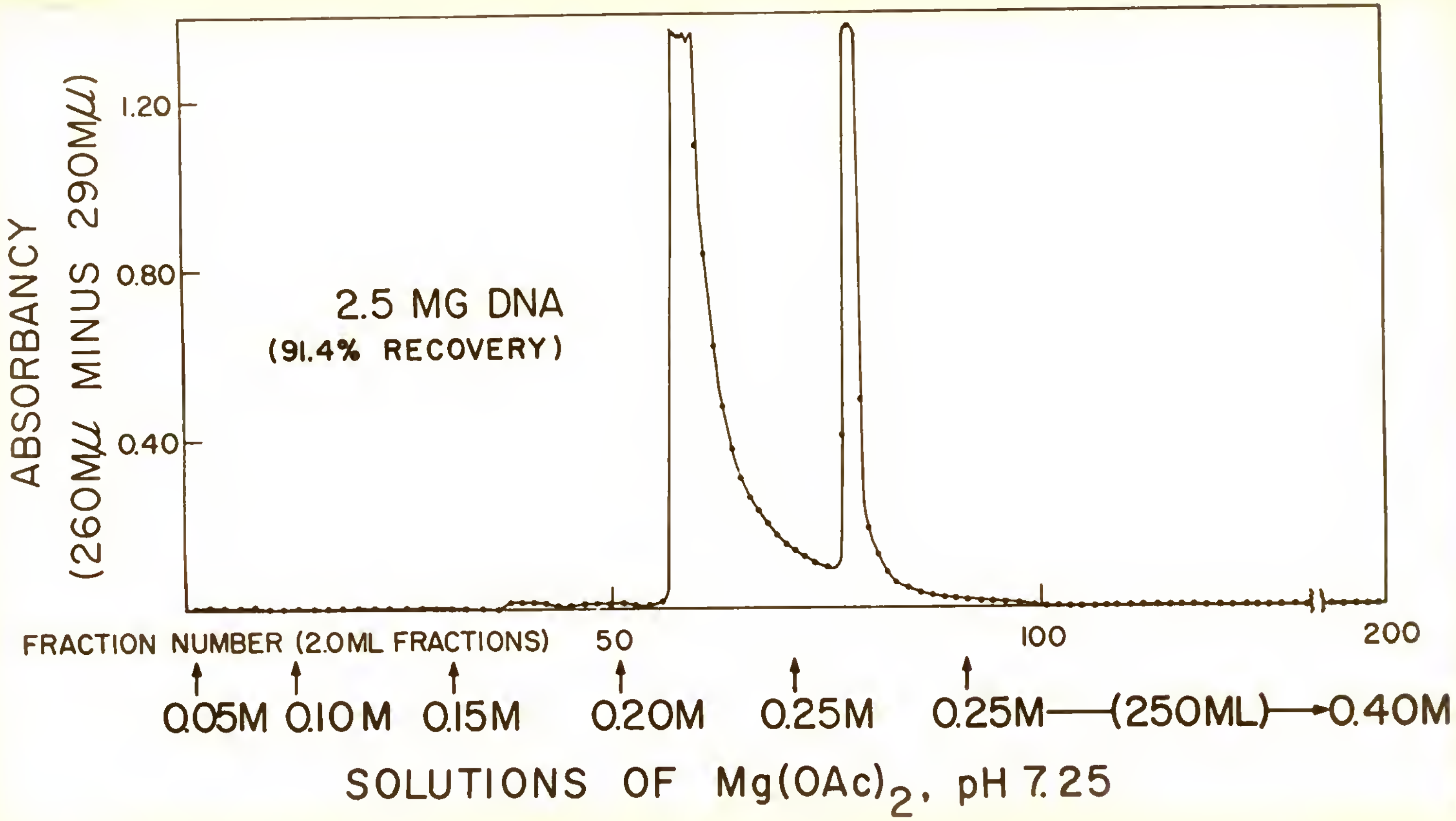

Fig. 3. Stepwise elution of deoxyribonucleate from a 30 x 0.9 cm column of Mg IRC-50, "through 325," at 30° C. The sample of deoxyribonucleate was from calf #32 thymus.

fractions recovered from the effluent in an experiment similar to that shown in Fig. 2B. The fractions were chosen so as to contain 20%, 40%, and 20% of the deoxyribonucleate applied to the column. As can be seen in Table II, the first and last fractions differed significantly in composition from the total preparation. These results indicate that the interaction of Mg IRC-50 with deoxyribonucleate is specific since reducing the capacity of the resin by increasing the $Mg(OAc)_2$ concentration causes a preferential elution of molecules rich in guanine and cytosine. That is, the affinity of the resin for molecules rich in adenine and thymine is greater than for molecules rich in guanine and cytosine. Accordingly, use of the interaction offers a means for separating molecules of deoxyribonucleate of different composition.

Capacity of Mg IRC-50 for deoxyribonucleate

A fundamental consideration in studies of the interaction of any adsorbent with a solute is the ultimate capacity of the adsorbent, since the effects of variables may be difficult to interpret if experiments are performed under conditions close to saturation. In order to estimate the maximum capacity of Mg IRC-50 for deoxyribonucleate, 73.4 ml of a 0.1% solution in 0.05 M $Mg(OAc)_2$ of the specimen from calf #32 thymus were added to a 28 x 2 cm column of "through 200" resin which had been equilibrated with 0.05 M $Mg(OAc)_2$, pH 7.2. The capacity of the resin was exceeded under these conditions, since deoxyribonucleate appeared in the effluent after about 80 ml had been collected. Unadsorbed nucleate was then removed from the column by irrigation with 0.05 M $Mg(OAc)_2$ until the absorbancy of the effluent

TABLE II

Base composition of successive fractions of deoxyribonucleate eluted from Mg IRC-50

The fractions were obtained from deoxyribonucleate from calf #30B thymus as noted in the text by a chromatographic experiment similar to that shown in Fig. 2B. The percentage in parentheses which follows each fraction number represents the portion of the deoxyribonucleate of the sample recovered in the respective fraction. The results are expressed as moles of base per 100 moles of total recovered bases. From Reference (20).

Base	Fraction number			Unfractionated, total deoxyribonucleate
	I(20%)	II(40%)	III(20%)	
Thymine	25.4	29.0	30.0	27.6
Guanine	24.2	21.7	20.6	22.7
Cytosine	23.6	20.2	19.7	22.3
Adenine	24.5	27.1	28.5	27.5
5-Methylcytosine	2.1	1.7	1.3	(1.4)

approached base line. The results of replicate experiments presented in Table III show that 1 cm^3 of packed resin binds about 0.3 mg of deoxyribonucleate.

As will be shown by the studies of base composition presented below, molecules of nucleate which are rich in guanine and cytosine have a low affinity for the resin and are eluted by 0.05 M $Mg(OAc)_2$ under the experimental conditions used to estimate the capacity of Mg IRC-50. When the extent of interaction is limited by the number of binding sites on the resin, molecules of deoxyribonucleate with the greatest affinity apparently compete with, and/or displace the less strongly bound molecules. Accordingly, the absolute capacity of the resin may well vary with the base composition or physical structure of the molecules tested. Deoxyribonucleate degraded by heat or by enzyme action may also interact differently with the resin. Moreover, particle size may affect the interaction, since equivalent amounts of smaller particles will present a greater area for the binding of macromolecules which are unable to penetrate the surface of the resin. The estimated maximum capacity of about 0.3 mg of deoxyribonucleate per cm^3 of Mg IRC-50 may be compared with 0.5 to 2.3 mg per cm^3 found for columns of ECTEOLA-cellulose (9). The value for the capacity of ECTEOLA-cellulose may include, however, entrained deoxyribonucleate which was eluted in the procedure used to estimate the capacity of Mg IRC-50 as described above.

In the experiments to be described, the amounts of deoxyribonucleate chromatographed were less than one-fourth, and usually about

TABLE III

Estimation of maximum capacity of Mg IRC-50 for deoxyribonucleate

In both experiments 28 x 2 cm columns which contained 88 cm^3 of packed resin were used. The sample was added as a 0.1% solution of deoxyribonucleate prepared in 0.05 M $Mg(OAc)_2$, pH 7.2.

Conditions	Experiment number	
	743	744
Deoxyribonucleate added		
Milligrams	74	75
Total absorbancy units	904	920
Deoxyribonucleate eluted by 0.05 M $Mg(OAc)_2$		
Milligrams	46	46
Total absorbancy units	560	564
Capacity		
Milligrams per column	28	29
Milligrams per cm^3 of packed resin	0.32	0.33

one-seventh of the estimated maximum capacity of the columns, except where "overload" experiments explicitly were performed.

Effect of certain variables on the interaction

Temperature -- The influence of temperature on the interaction between deoxyribonucleate and Mg IRC-50 is illustrated in Fig. 4. Deoxyribonucleate was eluted by a much lower salt concentration from the column operated at 30° C than from the column equilibrated and operated at 50° C. In contrast to the greater ease of elution customarily observed when low molecular weight compounds such as amino acids (28) and purines and pyrimidines[3] are chromatographed at higher temperatures, the deoxyribonucleate is more strongly bound by the Mg IRC-50 at the higher temperature. With small molecules, variations in temperature may affect ionizable groups of the chromatographed compound, of the buffer, and of the resin. The contribution of secondary valence forces to the overall interaction may also be altered. However, the effect of temperature on the chromatography of deoxyribonucleate using Mg IRC-50 appears to be dominated by other factors. The greater retardation observed at the higher temperature might best be explained in terms of effects on the physical structure of the resin or deoxyribonucleate. Heating may increase the number of effective binding sites per unit area of the surface of the resin by increasing the flexibility of the cross-linked polymer chains, thereby allowing the aliphatic matrix of the resin to swell. Higher temperatures may also reduce the degree of hydration of the two polymers, a factor which also would be expected to facilitate their mutual interaction. Elevated temperatures

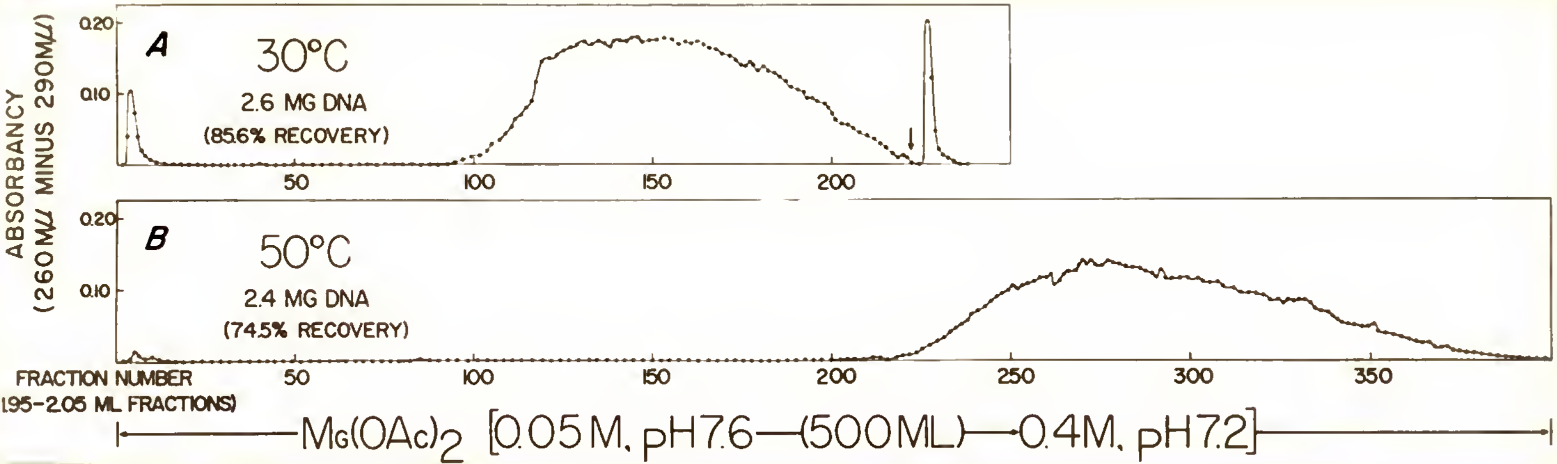

Fig. 4. Chromatography of deoxyribonucleate (A) at 30° C and (B) at 50° C by use of 29 x 0.9 cm columns of Mg IRC-50, "through 325." The sample of deoxyribonucleate used for (A) was obtained from calf #32 thymus, while the sample used for (B) was preparation 30A from calf thymus.

may induce still other structural alterations favorable to interaction such as changes in the extent of intramolecular hydrogen bonds in certain regions of the molecules of deoxyribonucleate.

pH -- The interaction between deoxyribonucleate and Mg IRC-50 is not appreciably sensitive to the precise value of pH of solutions of $Mg(OAc)_2$ over the range, pH 7.2 to pH 8.1, where most of the chromatographic experiments were performed. However, deoxyribonucleate passes without apparent retardation through columns of Mg IRC-50 which are equilibrated and operated with solutions of $Mg(OAc)_2$ of pH 6.1. A typical experiment performed at pH 6.1 is illustrated in Fig. 5A. It is reasonable to ascribe the absence of interaction at 0.05 M $Mg(OAc)_2$, pH 6.1, largely to the protonization of negative groups of the resin or the deoxyribonucleate or both. In this pH region, the carboxyl groups of Amberlite IRC-50 exhibit some buffering (29); the effect on the nucleate is probably limited to the secondary phosphoryl groups, whose pK is about 6.5 (30). It is possible that examination of the interaction at values of pH between 6.1 and 7.2, as well as at different molarities of $Mg(OAc)_2$, would permit a decision as to whether the observed pH effect depends more on changes in the nucleate than on changes in the resin.

Ions other than Mg^{++} -- In the interaction between IRC-50 and deoxyribonucleate, Mg^{++} may be regarded as a counter ion which at low concentrations is somehow responsible for the mutual affinity between both polymeric moieties. Higher concentrations of Mg^{++} destroy the specific interaction as indicated by elution of molecules with gradually varying composition. In order to determine whether deoxyribonucleate

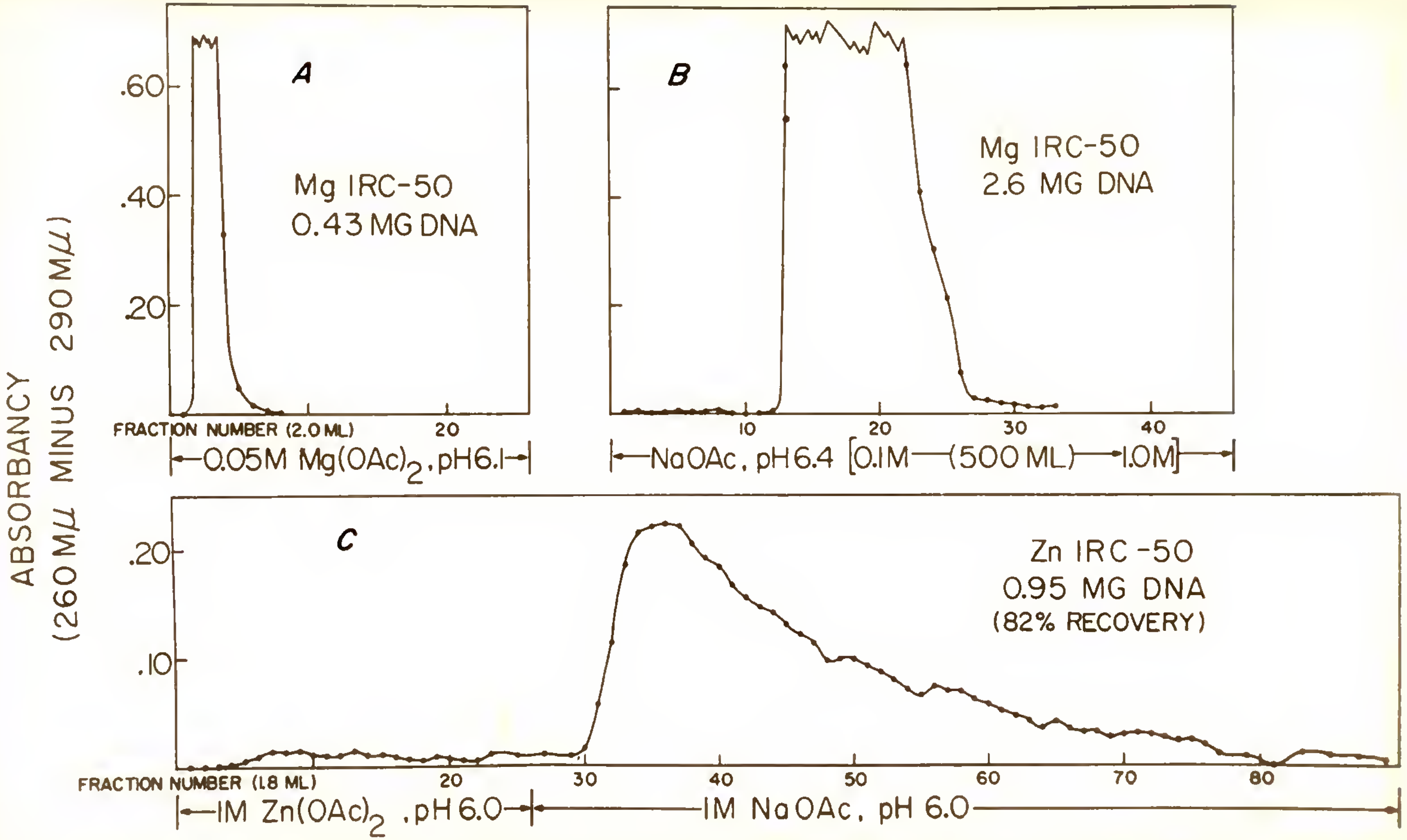

Fig. 5. The effect of certain variables on the chromatography of deoxyribonucleate from calf #32 thymus. The 29 x 0.9 cm columns of IRC-50, "through 200," used for the experiments were operated at 30° C. (A) shows that deoxyribonucleate was not adsorbed to a column of Mg IRC-50 equilibrated and operated with 0.05 M $Mg(OAc)_2$ of pH 6.1. (B) illustrates that deoxyribonucleate is eluted from columns of Mg IRC-50 by low concentrations of NaOAc. (C) shows that 1 M NaOAc, but not 1 M $Zn(OAc)_2$, elutes deoxyribonucleate from columns of Zn IRC-50.

can be eluted by other ions, a specimen from calf #32 thymus was adsorbed to a column of Mg IRC-50 under the usual conditions, but the eluent was produced by adding 4.7 M NaOAc, pH 7.0, to a 250 ml mixing chamber initially filled with 0.05 M $Mg(OAc)_2$, pH 7.3. The concentration of Na^+ originally chosen for the reservoir was high in order to compensate for the lower eluting power expected for a monovalent cation. However, 90% of the deoxyribonucleate was eluted by the time three hold-up volumes were collected. The unexpected prompt elution by NaOAc was confirmed by an otherwise similar experiment in which an eluent of pH 6.4 was produced by adding 1 M NaOAc to 0.1 M NaOAc. In this case (illustrated in Fig. 5*B*) 104% of the sample was recovered in less than two hold-up volumes. Thus, NaOAc, on a molar basis, was more than twice as effective as $Mg(OAc)_2$ in eluting deoxyribonucleate from Mg IRC-50. Although it remains to be determined whether elution with NaOAc will provide a series of fractions of gradually varying composition, it is noteworthy that elution from Mg IRC-50 by sodium salts has been utilized to effect a partial separation of deoxyribonucleates from *E. coli* and *Pneumococcus*.[6]

Although Na IRC-50 does not bind deoxyribonucleate at 0.2 M NaOAc, pH 6,[7] exploratory experiments have shown that divalent cations other than Mg^{++} are able to mediate the interaction between deoxyribonucleate and IRC-50.[8] Thus, deoxyribonucleate is bound by Ba IRC-50 at 0.1 M $Ba(OAc)_2$, pH 6.7, but is not eluted by 1 M $Ba(OAc)_2$, pH 6.[9] Similarly, all of the deoxyribonucleate in a solution of $Zn(OAc)_2$ was adsorbed to a column of Zn IRC-50 which had been equilibrated with 0.05 M $Zn(OAc)_2$, pH 6.0. It will be recalled, in contrast, that deoxyribonucleate was not bound by Mg IRC-50 at pH 6.1. Moreover, none of the deoxyribonucleate was eluted by 1 M $Zn(OAc)_2$ of pH 6.0 as shown in Fig. 5*C*. In view of the effectiveness of Na^+ in bringing about elution of deoxyribonucleate from Mg IRC-50, a solution of 1 M NaOAc, pH 6.0, was next passed through the column. As can be seen, 82% of the applied material was thereby recovered.

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[6] R. D. Hotchkiss and L. Mindich, personal communication (1959).

[7] C. F. Crampton, unpublished observations (1956).

[8] Unlike Mg IRC-50, the Mg form of Dowex 50-X2 (sulfonated polystyrene beads, "through 200" mesh) did not bind an appreciable amount

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of deoxyribonucleate at 0.05 M $Mg(OAc)_2$, pH 7.5. Similarly, the Mg form of Amberlite IR-120, XE-69 (crushed particles of sulfonated polystyrene, "through 325" mesh) did not bind the nucleate even when columns were operated at 0° C or at 5° C when the molarity of the $Mg(OAc)_2$ was reduced to 0.01 M. The Zn form of the XE-69 resin failed to bind deoxyribonucleate at 5° C when equilibrated with 0.05 M $Zn(OAc)_2$ of pH 6. These experiments were performed in an effort to find an ion exchanger for the chromatography of deoxyribonucleate which would equilibrate with the initial eluent more rapidly than IRC-50. However, the same factor responsible for the rapidity with which sulfonated polystyrene reaches equilibrium, namely, the predominantly ionic character of the linkage between sulfonate and cation, may underlie the inability of this resin to form a stable complex with deoxyribonucleate.

The Mg form of carboxymethyl-cellulose, a resin with reactive sites nearly identical to those of IRC-50, also failed to bind deoxyribonucleate when a column was operated with 0.05 M $Mg(OAc)_2$, pH 7.4, at 30°. It is possible that the carboxyl groups of carboxymethyl-cellulose are not disposed in a specific geometric configuration conducive to the formation of stable complexes with deoxyribonucleate. Moreover, carboxymethyl-cellulose lacks the hydrophobic matrix and methyl side chains which in IRC-50 may reinforce linkages involving divalent cations. The possible importance of the methyl groups attached to the hydrophobic matrix of IRC-50 is emphasized by an unpublished experiment of Crampton, Moore, and Stein (1956) which indicated that the Mg form of polyacrylic acid (Amberlite XE-112) did not bind deoxyribonucleate under conditions where polymethacrylic acid did.

[9] C. F. Crampton, S. Moore, and W. H. Stein, unpublished observations (1956).

The results discussed thus far suggest that under certain conditions, there is formed a ternary complex involving the negatively charged carboxyl groups of IRC-50, a divalent cation such as Mg^{++}, Zn^{++}, or Ba^{++}, and the negatively charged phosphate groups of deoxyribonucleate.[10] The observation that Zn IRC-50 binds nucleate at pH 6, while Mg IRC-50 does not, may be related to the fact that Zn^{++} forms stronger chelation complexes than Mg^{++} (31). If at certain critical salt concentrations, the bonds participating in complex formation are weakened, or salt becomes plentiful enough for the formation of stable binary complexes, the nucleate would be desorbed from the resin.

Whether a common mechanism, depending entirely on the ionic strength of the eluent, is responsible for elution by both NaOAc and $Mg(OAc)_2$, or whether there are additional effects such as cation binding, cannot as yet be decided. In any event, the desorption process appears to be specific and the cation concentration required for elution depends on the composition of the nucleate molecule.

It is reasonable to suggest that binding sites on deoxyribonucleate containing guanylic and cytidylic nucleotides, or in the vicinity of these residues, have a much lower affinity for Mg IRC-50 than other locations on the deoxyribonucleate polymer. Recent theoretical (32) and experimental (33) studies suggest that guanine-cytosine pairs have a higher electron density than adenine-thymine pairs. This could cause a preferential binding of cations such as Mg^{++} at regions that are abundant in guanine and cytosine. Such regions would thereby become less negatively charged, and consequently would exhibit less affinity for the resin than regions not bearing a Mg^{++}. Similar considerations may underlie the specific elution of deoxyribonucleate from other cationic adsorbants by sodium salts (5, 8) since Na^{+} may also be bound by deoxyribonucleate (34, 35). It is worth noting in this connection that low concentrations of $MgCl_2$ markedly reduced the concentration of NaCl required to elute deoxyribonucleate from histone-chloroform gels (5).

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[10] This interaction differs from that of Mg or Ba IRC-50 with histones (22) where the negatively charged carboxyl groups of the resin and the protonated side chains of these basic proteins are probably joined by electrostatic linkages. During the adsorption of histones, the cations originally present are presumably exchanged for the positively charged groups of lysine and arginine.

Flow rate -- For optimum resolution during the analysis of small organic compounds by ion exchange chromatography, the rate of elution must be commensurate with continuous equilibration between the adsorbed solute and the solute free in solution. However, most macromolecular substances are usually completely adsorbed to, or desorbed from, the chromatographic adsorbent. That is, the range of molarities (or pH, etc.) over which polymers will exhibit a finite distribution between the free and bound forms is small, and frequently the R_f of the compound will change abruptly from 0 to 1 (29). The suddenness of this change is suggestive of a cooperative phenomenon, and may in fact be viewed as deriving from the need to sever simultaneously the many bonds between the adsorbent and the multivalent macromolecule in order for the solute to be eluted.[11]

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[11] Because of this "all or none" phenomenon, the chromatographic behavior of many macromolecules is frequently independent of column length (29). This was found to be the case when deoxyribonucleate was chromatographed by use of a 72 x 0.9 cm column instead of the usual 30 x 0.9 cm column. No significant change in the chromatographic profile was observed except a slight, unexpected sharpening of the pattern.

Experiments were performed in order to determine the effect of flow rate on the behavior of deoxyribonucleate when chromatographed by use of Mg IRC-50. When deoxyribonucleate from calf thymus was chromatographed at flow rates varying from 4 ml per hour to 26 ml per hour, small but significant changes in the elution patterns were observed. At the lowest rate, there occurred a continuous elution of small amounts of ultraviolet absorbing material before the main portion of the nucleate emerged. However, elution of the bulk of the nucleate always required at least 0.2 M $Mg(OAc)_2$. If, therefore, the material leaching slowly from the column was deoxyribonucleate, it would represent a small amount of material in equilibrium with the bound nucleate at concentrations of $Mg(OAc)_2$ below 0.2 M. The effects of this equilibrium would be observable only at very low rates of flow.

In a subsequent section will be discussed the observation that a small amount of ultraviolet adsorbing material referred to as "heat peak" can be recovered from columns, after the greatest part of the deoxyribonucleate has been eluted, by raising the temperature of the column from 30° to 45° or 50° C, and simultaneously changing the eluent to 1 M $Mg(OAc)_2$. After the column had been operated at rates of 20 to 40 ml per hour, about 2 to 3% of deoxyribonucleate from calf thymus was recovered in this manner. However, at lower rates of flow, more material was recovered in the "heat peak," 4% at 7 ml per hour, and 9% at 4 ml per hour.

To determine if the length of time during which the sample remains adsorbed to the resin results in any physical or chemical change in the nucleate which might affect its chromatographic properties, 2.4 mg of the specimen from calf #32 thymus were applied to a column which was then held thirty-six hours before administration of the eluent. This delay had no apparent effect on the chromatographic pattern, except that 3.7% of the recovered ultraviolet absorption was eluted within one hold-up volume. It is difficult to assess the significance of this observation since, occasionally, small amounts of ultraviolet absorbing materials appear in this region of the chromatogram in the absence of any known modifications with respect to the sample or to the chromatographic procedure.

Particle size of resin -- When two chromatograms of deoxyribonucleate from calf thymus were run under conditions which were identical in all respects with the exception that one column was prepared from resin which had passed a 200 mesh screen, while the other was prepared from "through 325" mesh material, quite similar chromatographic profiles were obtained. It is possible that the higher proportion of small particles in the "through 325" material presents a greater effective surface area resulting in a higher capacity for the nucleate.

Chromatographic behavior of deoxyribonucleates from rat liver, Ehrlich ascites tumor cells, and Pneumococcus

The essential reproducibility of the chromatographic patterns given by specimens of deoxyribonucleate prepared from calf thymus by the several procedures noted in the experimental section has frequently been verified. It was of interest to determine if specimens from other sources likewise are adsorbed by Mg IRC-50 at low concentrations of $Mg(OAc)_2$, and if so, whether they can be eluted. The deoxyribonucleates of two other mammalian sources were studied. Figs. 8 and 10 show that specimens of deoxyribonucleate from rat liver and Ehrlich ascites tumor cells are, in fact, bound by Mg IRC-50 and can subsequently be eluted with an eluent of gradually increasing $Mg(OAc)_2$ concentration to provide chromatographic profiles very much like those obtained from calf thymus nucleate. The recovery of these three deoxyribonucleates, based on ultraviolet absorption, has usually been between 80% and 100%. When very small samples of deoxyribonucleate from rat liver were chromatographed by use of 30 x 0.9 cm columns, the recoveries were lower (62% and 75%).

A somewhat different result was obtained when the chromatographic behavior of the deoxyribonucleate from Pneumococcus was studied.[12] In the initial experiment, specimen 149 was chromatographed on a preparative column of Mg IRC-50 (Fig. 6A). This specimen possessed transforming activity and was estimated to contain 26% ribonucleate and 29% protein.[13] Of the total ultraviolet absorption applied to the column, 35% was unretarded at 0.05 M $Mg(OAc)_2$ and only 10% was eluted by means of the eluent of gradually increasing $Mg(OAc)_2$ concentration. It was found, however, that 10% of the ultraviolet absorption of the sample could be recovered from the resin in the "heat peak" by abruptly

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[12] We wish to express our appreciation to Dr. R. D. Hotchkiss for supplying us with several specimens of deoxyribonucleate from this source. The specimens were prepared in his laboratory by lysis of the bacterial cells with 0.15% deoxycholate, followed by several treatments with chloroform and isoamyl alcohol, as well as with ribonuclease.

[13] Performed by procedures to which reference is made in the manuscript noted in Footnote 1.

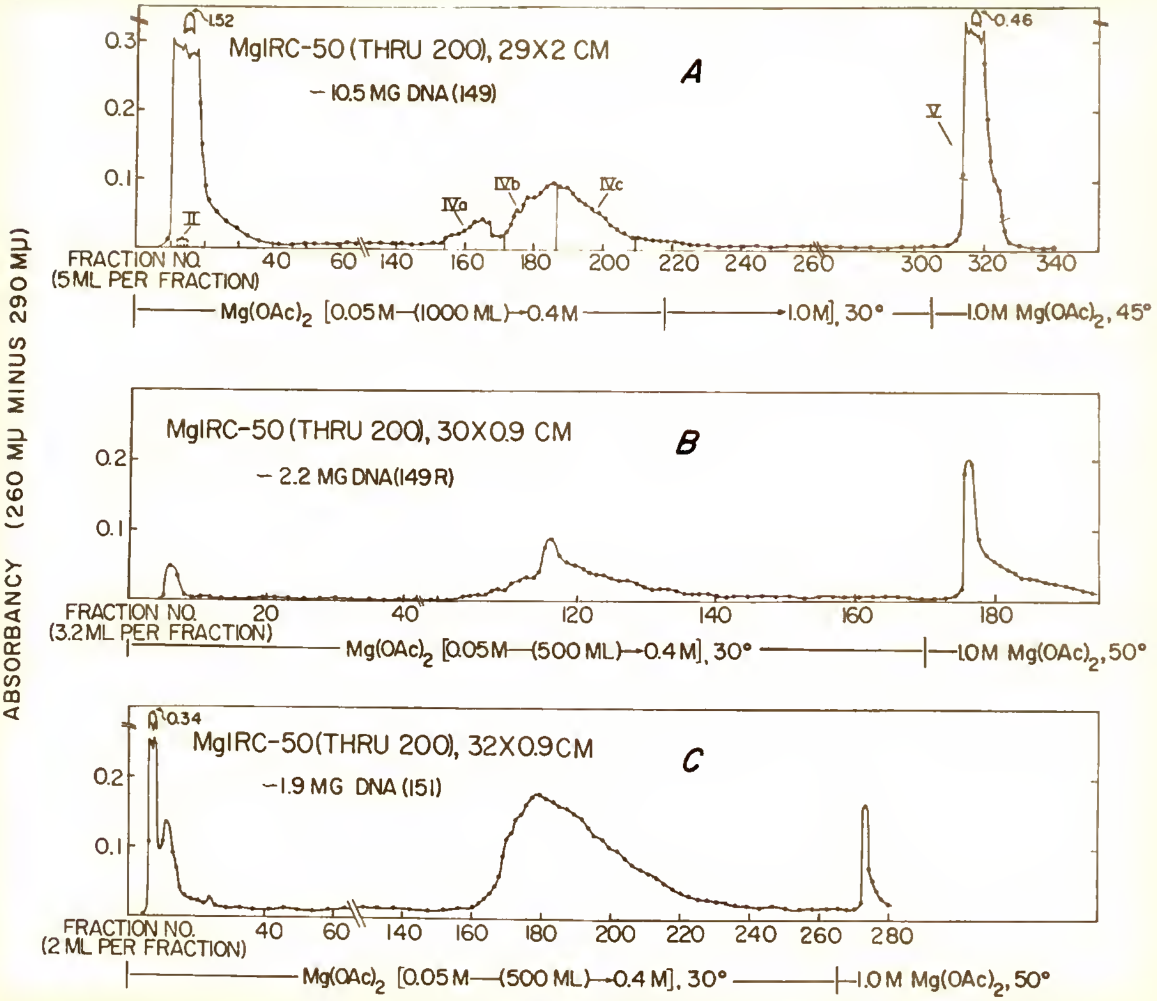

Fig. 6. Chromatography of specimens of deoxyribonucleate from *Pneumococcus* by use of columns of Mg IRC-50. Since slightly different conditions of elution were used for each experiment, the chromatograms have been plotted on different scales in order to facilitate a comparison of the relative distribution of ultraviolet absorbing materials. Specimens 149, 149R and 151 were used to obtain (*A*), (*B*) and (*C*), respectively.

increasing the temperature of the water jacketed column from 30° to 45° C and simultaneously applying 1 M $Mg(OAc)_2$. The analyses presented in Table IV suggest that the bulk of the contaminating ribonucleate was contained in material which passed directly through the resin (Fraction II), whereas protein was not confined to any single fraction. Results communicated to us by Dr. R. D. Hotchkiss indicate that while Fractions IVa, IVb, IVc, and V exhibit transforming activity, the unretarded material was devoid of such activity. Further purification of this same preparation by two treatments with chloroform and isoamyl alcohol yielded a product (149R) which on chromatography by use of an analytical column gave practically no unretarded constituents which absorbed ultraviolet light (Fig. 6B). The distribution of eluted material in the remaining portions of the chromatogram, however, remained unchanged. That is, both before and after repurification, 50% of the eluted deoxyribonucleate was found in the gradient elution region of the chromatogram, and 50% in the "heat peak" (Table V). Two additional pneumococcal specimens (152 and 152A), derived from the same original preparation, differed in their physical character when precipitated from solution in 0.85% NaCl by an equal volume of ethanol. Specimen 152 was fibrous, while 152A was jelly-like. When each of the samples was chromatographed, 36% of the total absorbancy eluted, exclusive of unretarded material, was present in "heat peak." Another preparation (151), however, yielded a chromatographic pattern which, as shown in Fig. 6C, was similar to that obtained from the mammalian nucleates, except for the presence of unretarded material.

TABLE IV

Distribution of constituents among chromatographic fractions of deoxyribonucleate from Pneumococcus

The sample chromatographed contained 130 absorbancy units, or about 10.6 mg of deoxyribonucleate. It gave orcinol color equivalent to approximately 2.6 mg of ribonucleate, and gave ninhydrin color equivalent to 2.9 mg of histone Fraction B from calf thymus. The orcinol and ninhydrin reactions were performed by procedures referred to in the manuscript noted in Footnote 1. The results are expressed as per cent of the constituent in the sample recovered in the fractions shown.

Constituent	Fraction number	
	II	V
Absorbancy units	21	10
Ribonucleate	51	4
Protein	20	24

TABLE V

Recovery of "heat peak" material from deoxyribonucleate preparations from various sources

Source	Preparation number	Per cent of added absorbancy units recovered in "heat peak"	Total column recovery[a]
Calf thymus	32	3	86
	32	3	96
Rat liver	11	5	92
	11	7	62
Ehrlich ascites tumor cells	7	3	88
	9	2	97
	16	3	105
Pneumococcus[b]	149	50	56
	149R	49	24
	151	6	67
	152	36	27
	152A	32	33

[a] Absorbancy units recovered as per cent of absorbancy units added.

[b] Because of wide variation of ribonucleate content and total recovery of absorbancy encountered with samples from *Pneumococcus*, the per cent recovered in "heat peak" is expressed, for these specimens, as per cent of the total absorbancy units eluted, exclusive of that which was unretarded on Mg IRC-50 at 0.05 M $Mg(OAc)_2$, pH 7.2 to 7.4.

As shown in Table V, 2 to 3% of the nucleate from calf thymus and ascites tumor cells, and 5 to 7% of that from rat liver is eluted in the "heat peak" position. Further studies should clarify whether the unusual chromatographic behavior of the pneumococcal deoxyribonucleates can be attributed to the use of preparative procedures very different from those employed in the isolation and purification of the nucleates from the other sources studied. In this connection, however, a specimen of calf thymus deoxyribonucleate which was prepared in the laboratory of Dr. R. D. Hotchkiss by two deproteinizations with chloroform and two treatments with Duponol, yielded a chromatographic pattern in all ways like that given by the specimens of deoxyribonucleate from calf #30 and #32 thymus glands. Additional evidence that "heat peak" material is not entirely an artifactitious product arising by action of the resin on the nucleate is provided by the observation that such material was absent from rechromatographed fractions of a specimen from rat liver (see Fig. 8B).[14] It must be recalled, however, that the amount of material in the "heat peak" increased simply by running the column at a very low rate of flow. Moreover, rechromatography of "heat peak" material from the nucleate of ascites tumor cells proved disappointing, since when a sample of 0.1 mg was applied to a fresh column of Mg IRC-50, nothing was eluted by the original conditions, and less than 10% was eluted by 1 M NaCl at 45° C. In this experiment, a significant amount of material was recovered only by passing 0.5 M NaOH through the column. Thus, while a part of the material in "heat peak" may be a genuine component of the nucleate specimen, a part may also be an artifact. In a subsequent section, which is concerned with studies of the incorporation of radioactive precursors into the deoxyribonucleate of Ehrlich ascites tumor cells, further observations regarding "heat peak" and its possible biological significance will be presented.

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[14] In one experiment, two fractions isolated by ethanol precipitation from an "overload" chromatogram of deoxyribonucleate from Ehrlich ascites tumor cells were combined and rechromatographed under non-overload conditions. One of the fractions contained material that originally emerged unretarded, while the other fraction contained material that was originally eluted gradually between 0.2 M and 0.4 M $Mg(OAc)_2$. On rechromatography, very little material emerged unretarded. However, about as much material was eluted in the "heat peak" as was eluted during gradient elution.

The "heat peak" material described here may be related to the fractions of deoxyribonucleate obtained from various sources by Brown and Brown (8). The fractions comprised 2 to 10% of the preparations, and were removed from columns of histone-cellulose by an eluent of high molarity and pH. These authors concluded that the material, which had a base composition similar to the original deoxyribonucleate, was an artifact produced by the column.

A characteristic feature of the chromatography of deoxyribonucleate from _Pneumococcus_ is the low recovery (24 to 67%) of ultraviolet absorbing material from columns of Mg IRC-50.

Rechromatography of fractions of deoxyribonucleate from calf thymus and rat liver

In order to study factors which determine the points of elution of molecules of deoxyribonucleate, material eluted at different regions of the $Mg(OAc)_2$ concentration gradient was adjusted to 0.05 M $Mg(OAc)_2$, combined, and rechromatographed on a fresh column of Mg IRC-50. Fig. 7_B_ shows an analytical chromatogram of two fractions of deoxyribonucleate which had been eluted from the preparative column illustrated in Fig. 7A and recovered by precipitation by ethanol. Similar experiments in which analytical columns were used both for isolation of fractions of deoxyribonucleate from rat liver and for their rechromatography are shown in Figs. 8A and 8B. In this case, the fractions had not been recovered for rechromatography by precipitation with ethanol, but were adjusted to 0.05 M $Mg(OAc)_2$ by dialysis. The results of the rechromatography experiments indicate that although there is some displacement in absolute position, the relative positions of elution are inherent properties characteristic of the molecules of deoxyribonucleate. That is, the gradual elution of successive fractions appears not to be governed by a heterogeneity of binding sites in the resin, or by a reduction in the capacity of the adsorbent to which all molecules of deoxyribonucleate are equally sensitive. It would be desirable to extend these experiments by rechromatographing mixtures of fractions isolated from radioactive and from non-radioactive specimens of deoxyribonucleate.

Effect of sample load

Although the relative positions of elution of the rechromatographed fractions of deoxyribonucleate from rat liver are similar to those of the original material (Fig. 8), the shift in absolute positions indicates that competition and displacement effects play a role in the fractionation process. The molecules in Fraction III of Fig. 8A must have the greatest affinity for the resin, since they require the

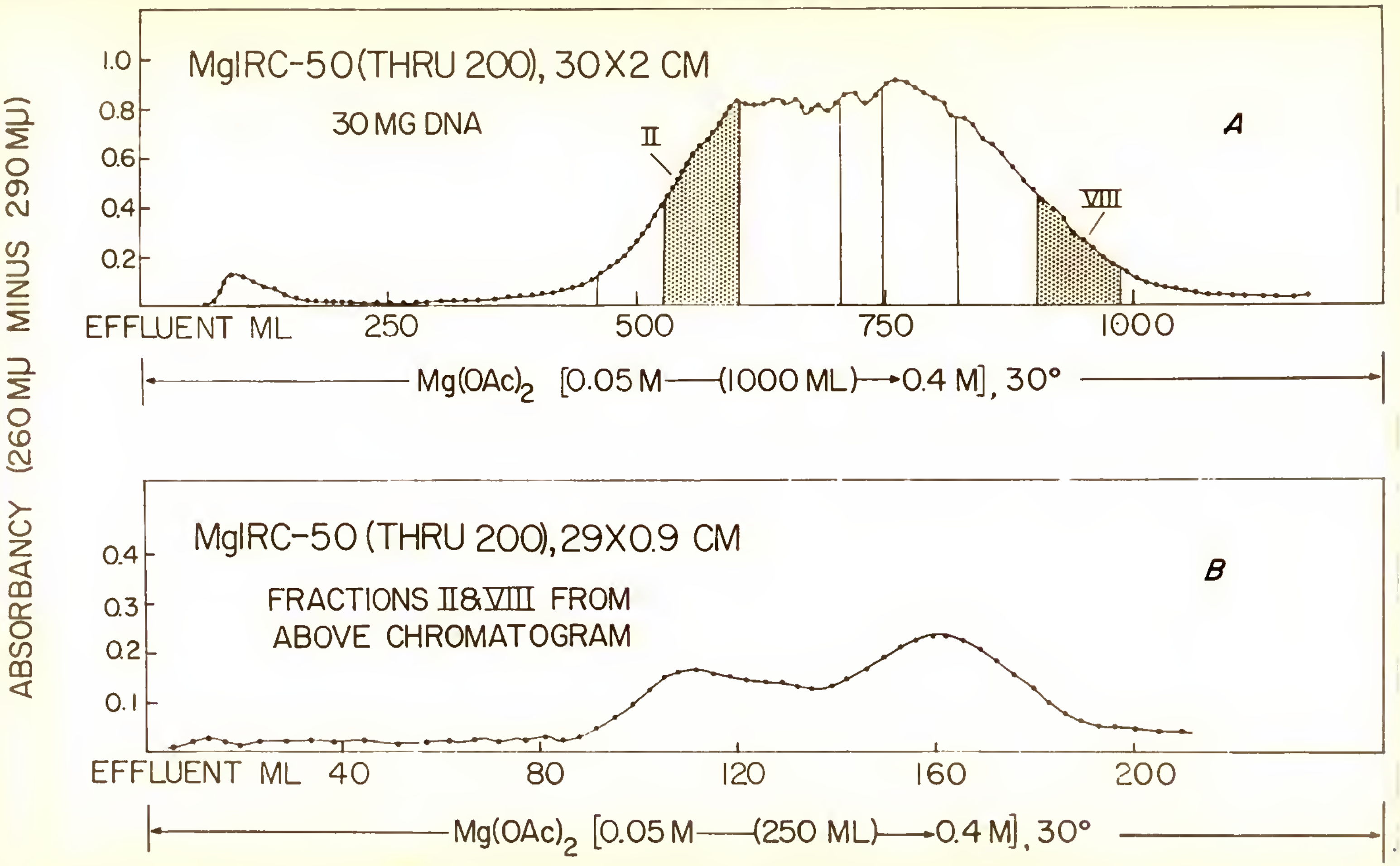

Fig. 7. Rechromatography of deoxyribonucleate from calf thymus by use of columns of Mg IRC-50, "through 200." The sample added to the 30 x 2 cm preparative column used to obtain (A) was specimen 30A from calf thymus. About 0.6 mg of Fraction II and 1.2 mg of Fraction VIII were mixed to provide the sample added to the 29 x 0.9 cm analytical chromatogram illustrated in (B).

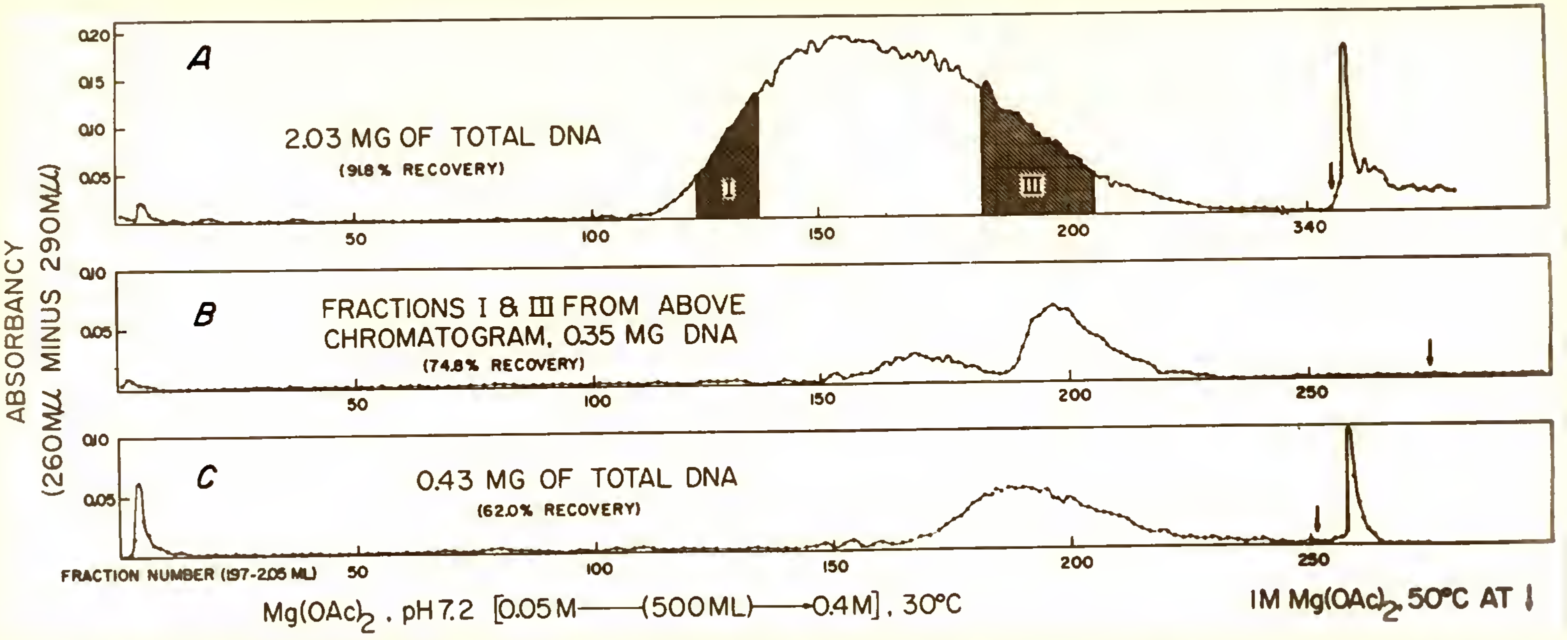

Fig. 8. Chromatography of deoxyribonucleate from rat liver by use of 29 x 0.9 cm columns of Mg IRC-50, "through 325." The total specimen from rat #11 liver was used to obtain (A) and (C), while a mixture of Fractions I and III from (A) served as the sample for (B).

highest salt concentration for their elution. Molecules having a strong affinity for Mg IRC-50 may be able to compete effectively for binding sites on the resin, and thereby to displace the less strongly bound molecules to positions further down the column. The positions of elution of different molecules of deoxyribonucleate may therefore be related to their initial positions on the column. When large samples are chromatographed, deoxyribonucleate may be adsorbed to the resin sites throughout most of the length of the column. Under such conditions, material might be expected to emerge earlier than when smaller samples are chromatographed. For example, in Fig. 8C, where 0.43 mg of deoxyribonucleate from rat #11 liver was chromatographed, elution did not begin until a higher $Mg(OAc)_2$ molarity was reached than in the experiment shown in Fig. 8A, where a 2 mg sample of the same specimen of deoxyribonucleate was chromatographed. Whether the concentration of deoxyribonucleate in the sample added to the column has an effect on the elution pattern has not been studied systematically. It must be noted that the sample solution used in Fig. 8A (1 mg per ml) was more concentrated than that used in Fig. 8C (0.2 mg per ml).

The possibility that deoxyribonucleate molecules having a low affinity for the resin are displaced by more strongly bound molecules was tested further as follows. Calf #32 thymus nucleate was added to columns of Mg IRC-50 in amounts sufficient to exceed the capacity of the resin. In the two experiments presented in Table VI, 62% and 36% of the specimens added were eluted under the starting conditions (0.05 M $Mg(OAc)_2$, pH 7.2). As shown, the material which emerged abruptly (Fractions I, II, and III of experiment 743 and Fractions I and II of

TABLE VI

Base composition of chromatographic fractions of deoxyribonucleate from calf #32 thymus obtained by overloading columns of Mg IRC-50

To the 28 x 2 cm column used for experiment 743 was added 74 mg of deoxyribonucleate, an amount which exceeded the capacity of the resin by 62%. Experiment 766 was performed by use of a 31 x 0.9 cm column to which was added 22 mg of deoxyribonucleate, an amount which exceeded the capacity of the resin by 36%.

Description of fractions	Experiment number						
	743				766		
Fraction number	I	II	III	IV	I	II	III
Per cent of deoxyribonucleate in fraction	10	11	8	28	18	18	52
Molarity of $Mg(OAc)_2$ used for elution	0.05	0.05	0.05	0.05	0.05	0.05	0.4
Bases[a]							
Thymine	25.2	25.9	--	27.3	25.7	27.0	28.7
Guanine	25.6	24.5	--	23.2	23.5	23.1	20.4
Cytosine	22.4	22.0	--	22.4	23.5	21.8	20.7
Adenine	24.5	26.2	--	25.5	25.1	26.1	29.2
5-Methylcytosine	2.5	1.5	--	1.6	2.2	2.0	0.9

[a] As moles of base per 100 moles of total recovered bases.

experiment 766) contained more guanine and cytosine than total deoxyribonucleate (see Tables II and VII), and thereby resembled deoxyribonucleate which is eluted first by the lower concentrations of $Mg(OAc)_2$ under "non-overload" conditions, such as Fraction I in Table II. On the other hand, the material not eluted by 0.05 M $Mg(OAc)_2$ but subsequently eluted as in experiment 766 by means of 0.4 M $Mg(OAc)_2$ has a composition like that of fractions eluted by the higher concentrations of $Mg(OAc)_2$ under "non-overload" conditions, such as Fractions II and III in Table II. It therefore seems likely that the same molecules of deoxyribonucleate which are eluted early by increasing concentrations of $Mg(OAc)_2$ from a column bearing a small load of deoxyribonucleate, are among the first to emerge when the capacity of a column is deliberately exceeded. Even when the sample load is excessive, interaction of Mg IRC-50 with deoxyribonucleate remains demonstrably specific.

Base composition of fractions of deoxyribonucleate from calf thymus and Ehrlich ascites tumor cells

As discussed above, the interaction of deoxyribonucleate with Mg IRC-50 has provided a means for eluting fractions of different composition from columns to which specimens of total deoxyribonucleate were adsorbed. The studies with overloaded columns suggested an equally effective alternative fractionation technique, as discussed in connection with Table VI. A more complete chromatographic fractionation of deoxyribonucleate from calf #32 thymus is illustrated in Fig. 9. The base compositions of fractions of the unadsorbed deoxyribonucleate and of the fractions eluted gradually by increasing concentrations of $Mg(OAc)_2$ are shown in Table VII.

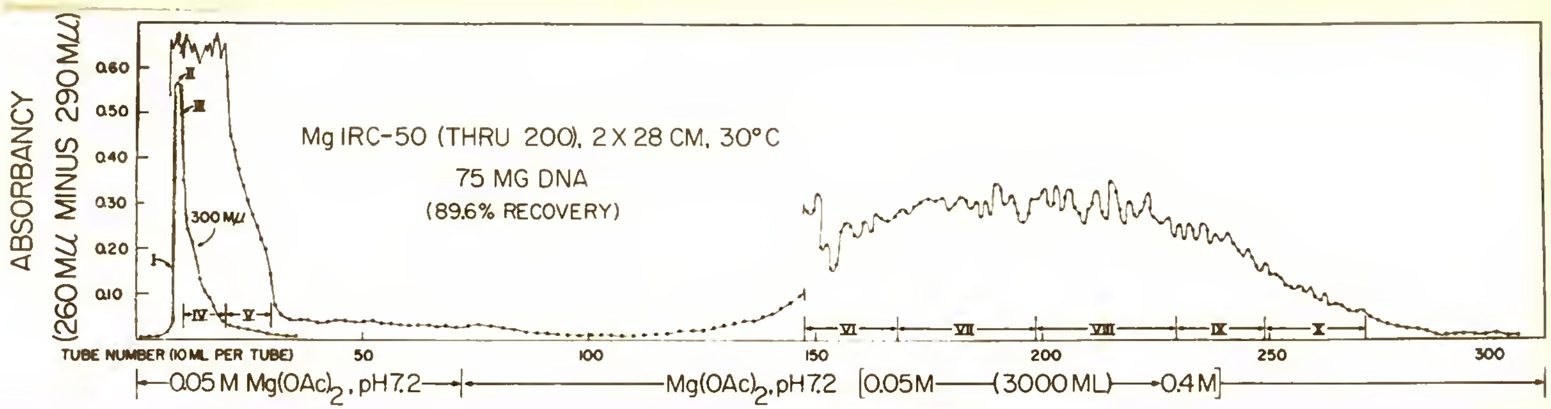

Fig. 9. Preparative chromatography of deoxyribonucleate from calf #32 thymus by use of overload conditions. In the experiment shown, the capacity of the 28 x 2 cm column of Mg IRC-50 was exceeded so that 59% of the added deoxyribonucleate was eluted by 0.05 M Mg(OAc)$_2$. The nominal temperature at which the column was operated was 30° C. The unusual fluctuations in the elution pattern are probably the result of irregular variations which were greater than ± 0.2° C.

TABLE VII

Base composition of chromatographic fractions of deoxyribonucleate from calf #32 thymus

The fractions were derived from the experiment illustrated in Fig. 9. The percentage in parentheses which follows each fraction number represents the portion of the deoxyribonucleate of the sample recovered in the respective fraction. The results are expressed as moles of base per 100 moles of total recovered bases.

Base	Fraction number										Unfractionated total deoxyribonucleate
	I(3%)	II(12%)	III(11%)	IV(29%)	V(4%)	VI(5%)	VII(9%)	VIII(10%)	IX(4%)	X(2%)	
Thymine	23.3	26.3	27.4	28.3	25.3	30.3	30.7	30.3	30.4	32.1	27.9
Guanine	27.1	24.2	23.3	22.0	25.0	20.4	20.1	20.0	19.4	19.6	22.8
Cytosine	23.5	22.6	22.1	21.3	22.1	19.8	18.9	19.0	19.2	18.1	21.1
Adenine	22.9	25.0	25.3	26.4	25.3	28.6	29.4	29.9	30.3	29.7	26.8
5-Methylcytosine	3.1	1.9	2.0	2.0	2.3	0.9	0.9	0.8	0.7	0.6	1.5
$\frac{A + T^{a}}{G + C + MC}$	0.86	1.05	1.11	1.20	1.02	1.44	1.51	1.51	1.54	1.62	1.21

[a] The ratio of the molar quantities of adenine plus thymine to guanine plus cytosine plus 5-methylcytosine.

The successive fractions show variations in composition similar to fractions previously obtained by Chargaff, Crampton, and Lipshitz (4, 5) and by Brown and Brown (8), whose methods were based on the interaction of deoxyribonucleate with basic proteins. Chargaff *et al.* noted that the ratio of 5-methylcytosine to cytosine in the successive fractions varied markedly, and concluded that the replacement of cytosine by the 5-methyl derivative does not occur at random. As shown in Table VII, a 5-fold change in the 5-methylcytosine content of the chromatographic fractions occurs with less than a doubling of cytosine or guanine, which confirms the results of Chargaff *et al.*

Chromatograms of deoxyribonucleate from Ehrlich ascites tumor cells obtained under "non-overload" conditions (EA #1) or overload conditions (EA #7) are shown in Figs. 10 and 11, respectively. The base compositions of the isolated fractions are presented in Tables VIII and IX. Both techniques yielded fractions which show the same trends in composition as the fractions from calf thymus deoxyribonucleate. However, the extreme fractions of the deoxyribonucleate from tumor cells differ far less than the corresponding fractions shown in Tables II, VI, and VII.

Incorporation of Precursors into Fractions of Deoxyribonucleate from Ehrlich Ascites Tumor Cells

Preliminary experiments with C^{14}-formate

The ability of Mg IRC-50 to separate chemically different molecules of deoxyribonucleate suggested that information regarding the synthesis of these molecules might be provided by studies of the initial

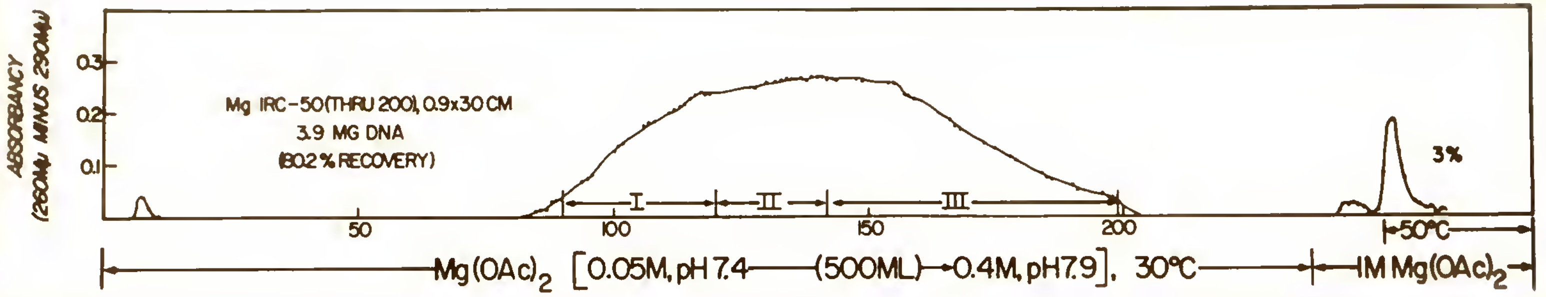

Fig. 10. Chromatography of deoxyribonucleate from Ehrlich ascites tumor cells (EA #1) by use of Mg IRC-50.

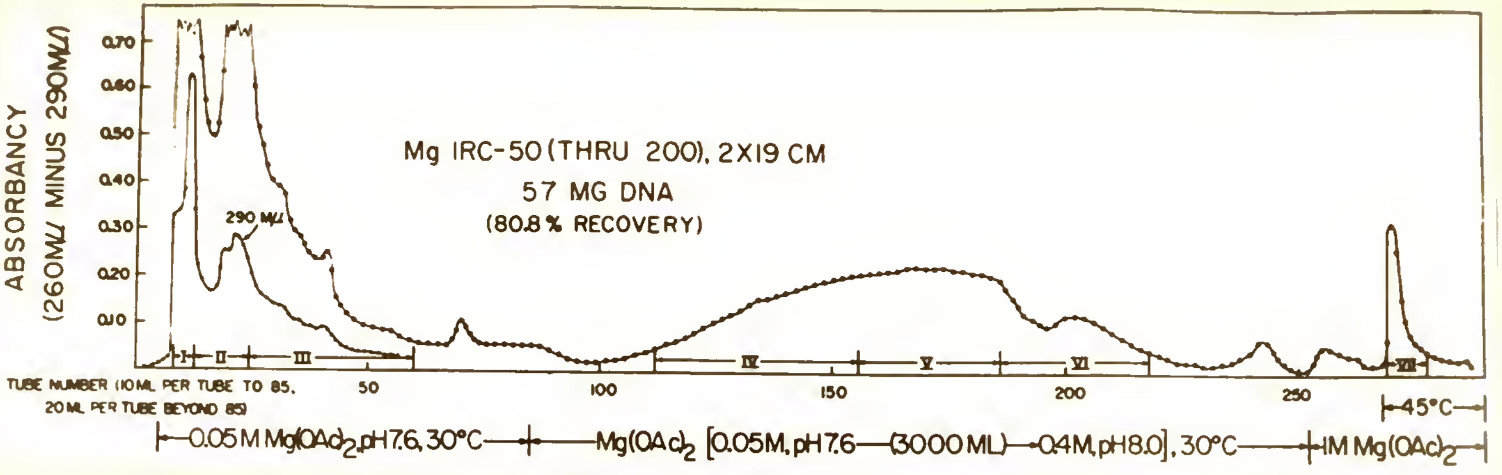

Fig. 11. Preparative chromatography of deoxyribonucleate from Ehrlich ascites tumor cells (EA #7) by use of overload conditions. In the experiment shown, the capacity of the 19 x 2 cm column of Mg IRC-50 was exceeded so that 32% of the added deoxyribonucleate was eluted by 0.05 M $Mg(OAc)_2$. Discontinuous changes in rate of flow were responsible for the abrupt changes in the curvature of the elution pattern at tube numbers 70, 190, and 240.

TABLE VIII

Base composition of chromatographic fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #1)

The fractions were derived from the experiment illustrated in Fig. 10. The percentage in parentheses which follows each fraction number represents the portion of the deoxyribonucleate of the sample recovered in the respective fraction. The results are expressed as moles of base per 100 moles of total recovered bases.

Base	Fraction number			Unfractionated, total deoxyribonucleate
	I(19%)	II(24%)	III(34%)	
Thymine	27.7	28.1	29.2	28.8
Guanine	22.2	21.1	20.6	21.3
Cytosine	21.0	21.7	19.6	19.5
Adenine	28.8	28.8	30.0	29.5
5-Methylcytosine	(0.4)	(0.3)	0.7	0.9
$\frac{A + T}{G + C + MC}$[a]	1.30	1.32	1.45	1.40

[a] The ratio of the molar quantities of adenine plus thymine to guanine plus cytosine plus 5-methylcytosine.

TABLE IX

Base composition of chromatographic fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #7)

The fractions were derived from the experiment illustrated in Fig. 11. The percentage in parentheses following each fraction number represents the portion of the deoxyribonucleate of the sample recovered in the respective fraction. The results are expressed as moles of base per 100 moles of total recovered bases.

Base	Fraction number							Unfractionated, total deoxyribonucleate
	I(9%)	II(12%)	III(11%)	IV(17%)	V(19%)	VI(10%)	VII(3%)	
Thymine	27.2	28.2	27.9	28.8	29.6	30.7	29.0	28.4
Guanine	22.7	22.1	22.2	21.2	20.4	20.7	21.3	20.5
Cytosine	21.5	21.1	21.6	20.2	19.3	17.3	19.3	20.9
Adenine	27.4	27.9	27.6	29.2	29.8	30.2	29.4	29.3
5-Methylcytosine	1.1	0.7	0.7	0.6	0.9	1.2	1.0	0.9
$\frac{A + T^a}{G + C + MC}$	1.21	1.28	1.25	1.38	1.46	1.55	1.40	1.36

[a] The ratio of the molar quantities of adenine plus thymine to guanine plus cytosine plus 5-methylcytosine.

in vivo rates of incorporation of radioactive precursors. Cultures of Ehrlich ascites tumor cells growing in the peritoneal cavities of mice were chosen for these studies because they present the following advantages. *1*) During the logarithmic phase of growth under natural *in vivo* conditions, the cells are less likely to lose viability than cells grown *in vitro*. Consequently, the possibility of an abnormal metabolism of their deoxyribonucleate is minimized and most findings can be construed with reasonable confidence to have a bearing on the synthesis of authentic deoxyribonucleate. *2*) Populations of ascites tumor cells are relatively homogeneous in contrast to alternative biological systems in which there is rapid synthesis of deoxyribonucleate such as regenerating liver. The interpretation of any chemical or metabolic properties of fractions of the deoxyribonucleate of liver would be confused by the presence of several cell types which respond differently to hepatectomy, as was recently found by Edwards *et al*. (36). To be sure, *in vivo* cultures of Ehrlich ascites tumor cells gradually become hemorrhagic. Although absent from erythrocytes, deoxyribonucleate is present in leucocytes, which may also infiltrate the cultures, but which rarely, if ever, exceed about 1% of the cell population. *3*) Finally, of great practical importance, is the fact that deoxyribonucleate is readily obtained from these cells in good yield and in a highly polymerized form by use of strong urea solutions.[1] The denatured deoxyribonucleate provided by certain alternative extraction procedures (37) would not be suitable for fractionation by use of Mg IRC-50, although they could contain constituents absent from the specimens examined in the present studies.

Effect of non-radioactive formate -- After the administration of C^{14}-formate, it was necessary to allow time enough for a measurable amount of radioactivity to be incorporated into deoxyribonucleate. It was desired, however, to limit the duration of the incorporation period, so that only a small proportion of the total deoxyribonucleate would have the opportunity to replicate in the presence of the precursor. A brief period would tend to exaggerate any preferential uptake of the precursor into chromatographically different fractions. An incorporation period of ten minutes was chosen for the first experiment. This corresponds to about 1% of the time required for the cells to double in number (eighteen hours). The C^{14}-formate injected is an efficient precursor of the 2 and 8 positions of the purine ring, and of the 5-methyl group of thymine. With these considerations in mind, the ten minute incorporation period was followed by an interval of 110 minutes in order to allow time for incipiently labeled molecules of deoxyribonucleate to be completed, to undergo secondary structural alterations, to combine with histone, or to acquire whatever other characteristics may be essential for insolubility in the urea medium used during isolation. In an effort to minimize further incorporation during the 110 minute interval, a 100-fold excess of unlabeled formate was injected ten minutes after the C^{14}-formate. It was anticipated that this amount of unlabeled formate would dilute the labeled pools of the cells so that the radioactivity incorporated during the 110 minute interval would be insignificant compared with that incorporated in the preceding ten minutes.

Accordingly, each of two tumor-bearing mice (EA #1) was injected with 0.1 ml of buffered saline solution containing 5 μcuries of C^{14}-formate having a specific activity of 1 μcurie per μmole. The mice were reinjected after ten minutes with 1 ml of buffered saline solution containing 500 μmoles of unlabeled formate. The tumor cells were harvested 120 minutes after the injection of C^{14}-formate, and were processed as described in the experimental section. The twice-deproteinized deoxyribonucleate was found to contain appreciable amounts of carbon-14 in thymine, guanine, and adenine. Cytosine contained no appreciable radioactivity, while 5-methylcytosine was found to contain some counts, which, however, could have resulted from contamination by the highly labeled adenine as a result of the incomplete separation of these two bases (see Fig. 1). Therefore, it is still not known whether formate is a direct precursor of the methyl group of 5-methylcytosine. A 3.9 mg specimen of the deoxyribonucleate was eluted from an analytical column of Mg IRC-50 (30 x 0.9 cm) by use of an eluent of gradually increasing $Mg(OAc)_2$ concentration (0.05 M to 0.4 M) to obtain the chromatogram shown in Fig. 10. The effluent was combined to provide three successive fractions from which the nucleate was precipitated by ethanol and hydrolyzed. The hydrolysates were analyzed by use of columns of Dowex 50 in order to estimate the base composition of the fractions and to separate the bases for the subsequent determination of specific activities. The base compositions have already been presented in Table VIII. Specific activities are given in Table X. Under the conditions of incorporation used in this experiment, it is apparent that all of the fractions attained similar specific activities.

TABLE X

In vivo incorporation of C^{14}-formate into fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #1)

The fractions were derived from the experiment illustrated in Fig. 10. The percentage in parentheses which follows each fraction number represents the portion of the deoxyribonucleate of the sample recovered in the respective fraction. The results are expressed as counts per minute per μmole of base.

Base	Fraction number			Unfractionated, total deoxyribonucleate
	I(19%)	II(24%)	III(34%)	
Thymine	372	400	404	422
Guanine	286	236	281	256
Adenine	226	191	195	204

Effect of time -- In view of the results of the preceding experiment, three additional experiments were performed in order to evaluate the efficiency with which the 500 μmoles of unlabeled formate prevent further incorporation of C^{14}-formate into the tumor cell nucleate. In all three experiments, the results of which are presented in Fig. 12, an interval of seven minutes was allowed to elapse after C^{14}-formate. However, the period of exposure to unlabeled formate was varied. In the first experimental group of three mice (EA #4), this period was 113 minutes. That is, 500 μmoles of unlabeled formate were injected to each mouse seven minutes after 5 μcuries of C^{14}-formate had been injected, and the tumor cells were isolated 120 minutes after the injection of C^{14}-formate. In the second group of four mice (EA #5), C^{14}-formate and the unlabeled compound were injected as for EA #4, but the cells were isolated twenty-five minutes after the injection of C^{14}-formate. In the third group of three mice (EA #6), unlabeled formate was not injected at all. That is, the tumor cells were isolated promptly seven minutes after injection of C^{14}-formate. If the administration of unlabeled formate completely and immediately prevents further uptake of the labeled precursor, the specific activity of the bases in the deoxyribonucleate isolated from all three groups of mice would be the same or possibly would decrease with time because of the continued synthesis of deoxyribonucleate from diluted pools of precursors. As shown in Fig. 12, however, the incorporation of radioactivity into the purines continued slowly for 120 minutes despite the injection of a 100-fold molar excess of unlabeled formate. On the other hand, the specific activity of thymine did remain the same over

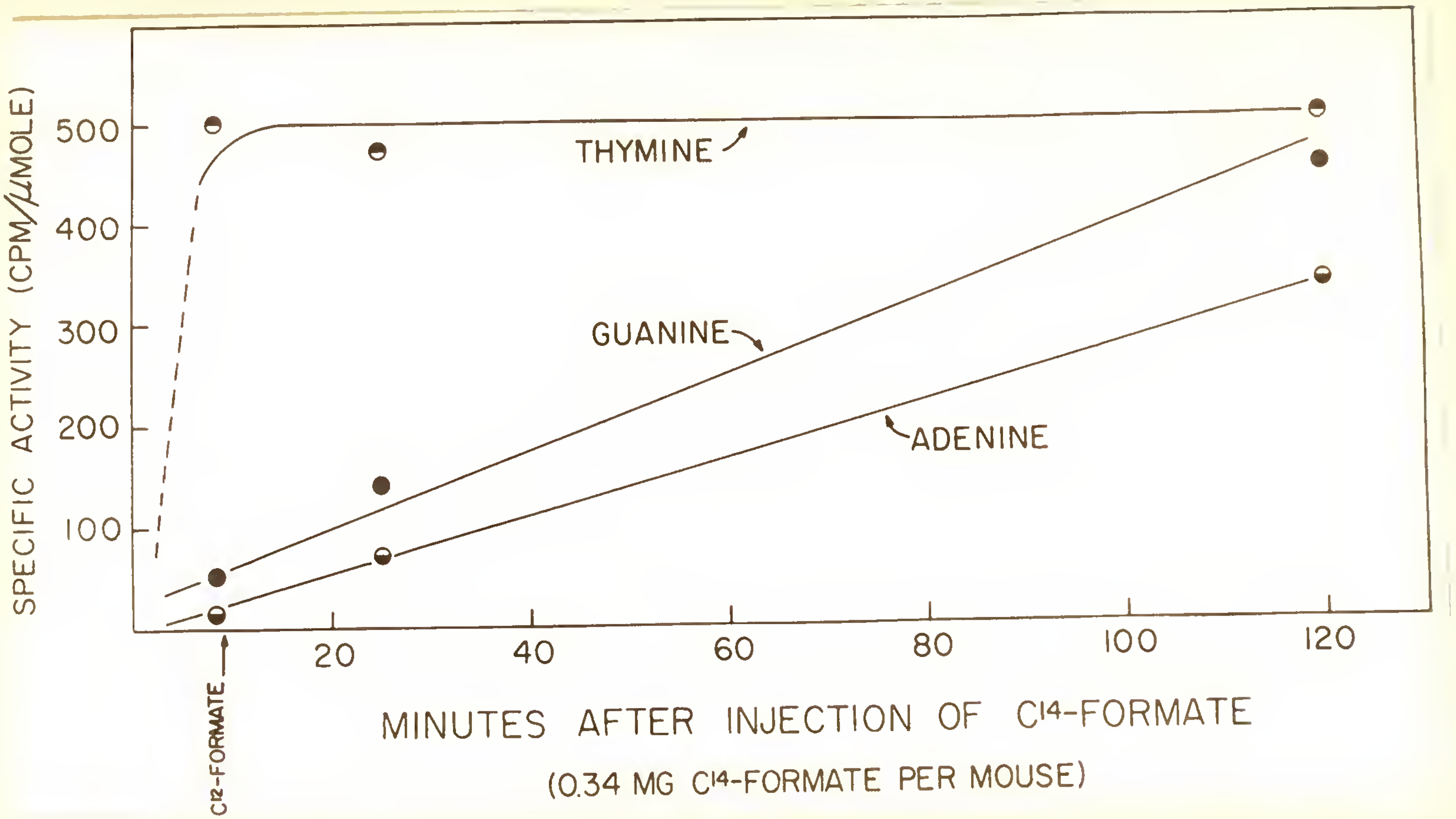

Fig. 12. Effect of time of incorporation of C^{14}-formate on the specific activity of bases of deoxyribonucleate from Ehrlich ascites tumor cells. The points at 7, 25 and 120 minutes correspond to data for specimens EA #6, 5 and 4, respectively.

the intervals studied, suggesting that the non-radioactive formate injected did succeed in diluting pools of precursors of this base. The differences among the curves obtained for the purines and for thymine could result from differences in the size of the pools of the respective precursors. Thus, the purines may be formed from large pools with which formate equilibrates slowly and incompletely, while thymine may be formed from small pools with which formate equilibrates rapidly and more completely.

Incorporation of C^{14}-formate into fractions of deoxyribonucleate

As noted above, deoxyribonucleate containing reasonably large amounts of radioactive thymine is recoverable from tumor cells seven minutes after the injection of C^{14}-formate. Since deproteinized deoxyribonucleate is soluble in the urea medium, this observation suggests the possibility that deoxyribonucleate may be insoluble in the urea medium even during the initial stages of replication. This observation is perhaps related to the recent finding that histones are conserved as well as deoxyribonucleate during replication of the tumor cells (38). In this system, therefore, deoxyribonucleate at all stages of replication may be insoluble in the urea medium because it occurs combined with histone. At any rate, the finding indicated that studies of the heterogeneity in the uptake of precursors into fractions of deoxyribonucleate during even shorter intervals were feasible. Under these conditions, preferential labeling would be accentuated by the rapidity with which the specific activity of the pools of precursors of thymine changes.

In the next experiment, therefore, tumor cells (EA #7) were collected from each of ten mice five minutes after the injection of 0.1 ml of buffered saline containing 3.7 μmoles of labeled formate with a specific activity of 13.7 μcuries per μmole. A fifty-seven mg sample of deoxyribonucleate isolated from the cells was chromatographed by use of a preparative column of Mg IRC-50 (19 x 2 cm), with the results shown in Fig. 11. Under these overload conditions, 32% of the sample was eluted by 0.05 M $Mg(OAc)_2$. Most of the remaining deoxyribonucleate was removed from the column by gradient elution with $Mg(OAc)_2$ (0.05 M to 0.4 M). A further small portion, referred to as "heat peak," was eluted by 1 M $Mg(OAc)_2$ when the temperature of the column was raised to 45° C.

The column effluent was combined to provide seven fractions. The deoxyribonucleate which was recovered from the fractions by precipitation with ethanol was found to have the base compositions shown in Table IX. As discussed earlier, the extreme fractions differ far less than extreme fractions which have been obtained by similar methods from deoxyribonucleate from calf thymus.

Despite the similar base compositions, however, significant differences were found with respect to the specific activity of the thymine in these fractions, as shown in Table XI. The first three fractions comprise the nucleate eluted by 0.05 M $Mg(OAc)_2$ under the overload conditions. The specific activity of the thymine in these three fractions is higher than that in the next three fractions which contain material recovered from the column by gradient elution. The thymine present in Fraction VII, the "heat peak," was found to have the

TABLE XI

Five minute in vivo incorporation of C^{14}-formate into the thymine of fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #7)

The fractions were derived from the experiment illustrated in Fig. 11. The percentage in parentheses which follows each fraction number represents the portion of the deoxyribonucleate of the sample recovered in the respective fraction. The results are expressed as counts per minute per µmole of thymine.

Method of isolation of thymine from hydrolysate	Fraction number							Unfractionated, total deoxyribonucleate
	I(9%)	II(12%)	III(11%)	IV(17%)	V(19%)	VI(10%)	VII(3%)	
Column chromatography	697	773	651	488	332	639	2870	857
Rechromatography on paper following column chromatography	844	818	741 ± 16[a]	516	388	578	2960	938 ± 9[a]
Direct chromatography on paper		830 ± 10[a]			434 ± 1[a]			926 ± 0[a]

[a] The variation between duplicate determinations.

highest specific activity. While the fractions shown account for 88% of the absorbancy applied to the column, they account only for 60% of the radioactivity. The discrepancy would be accounted for if material having a specific activity of about 3000 CPM/μmole (a value similar to that of Fraction VII) remained on the column.

The observed differences in specific activity could result from contamination of the thymine by constituents arising from the destruction of deoxyribose by formic acid, or from hydrolysis to amino acids of variable amounts of protein or other contaminants in the successive fractions of deoxyribonucleate. Acidic decomposition products would probably emerge near thymine from columns of Dowex 50.[15] In order to explore this possibility, the thymine residues were eluted from the planchets with 0.1 N HCl and rechromatographed on filter paper by use of isopropanol-HCl. The specific activities of the thymine after rechromatography on paper, also presented in Table XI, were quite similar to those determined directly on the Dowex 50 effluent. The slight increase observed after rechromatography may have resulted from the elimination of materials which contributed ultraviolet absorption.[5] In order to determine whether reproducible values for specific activities could be obtained by use of paper chromatography alone, independent hydrolysates of aliquots of Fractions II and V, and of the total deoxyribonucleate were chromatographed directly, and analyzed as before. As shown in Table XI, the duplicate determinations agreed within less than

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[15] The sulfuric acid in which the hydrolysate of the deoxyribonucleate was dissolved prior to chromatography would presumably be excluded from the polysulfonate resin; in fact, it was found to be eluted in about 24 ml, while thymine emerged after about 60 ml.

2.5%, and were similar to the values originally found. Appropriate analyses of other regions of such paper chromatograms provided values for the specific activities of adenine and guanine of some of the fractions. As shown in Table XII, the specific activity of guanine was found to vary among the fractions in the same way as that of thymine.

The evidence strongly suggests that there are present in the specimen of deoxyribonucleate described above, molecules which vary not only in base composition but also in the extent to which they incorporated radioactivity from C^{14}-formate during the five minute in vivo incubation period. That such variations in the extent of labeling can be obtained reproducibly was confirmed by isolating deoxyribonucleate from tumor cells removed from each of five of a second group of mice (EA #9) five minutes after the injection of 50 μcuries of C^{14}-formate. A solution containing 12.6 mg of the specimen was added to an analytical column of Mg IRC-50 (28 x 0.9 cm). Under these overload conditions, 45% of the sample was eluted from the resin by 0.05 M $Mg(OAc)_2$. As shown in Fig. 13, stepwise changes of the eluent provided 47% of the sample at 0.4 M $Mg(OAc)_2$, and 2% at 1 M $Mg(OAc)_2$ when the temperature was raised to 50° C. A small amount of additional material was eluted finally by 0.5 M NaOH at room temperature. The specific activities of the thymine of the four fractions were determined after paper chromatography and are shown in Table XIII. The pattern of differential labeling is very similar to that found in the previous experiment, despite the examination of fewer, larger fractions in the present case.

TABLE XII

The specific activity of bases in fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #7)

The fractions were derived from the chromatogram illustrated in Fig. 11. To obtain the results shown here, the bases were isolated from hydrolysates by chromatography on paper, either directly, or after initial purification by use of columns of Dowex 50.

Fraction	Specific activity (CPM/μmole)		
	Adenine	Guanine	Thymine
II	18	46	818
V	--	12	388
VII	--	202	2960
Unfractionated, total deoxyribonucleate	28	63	938

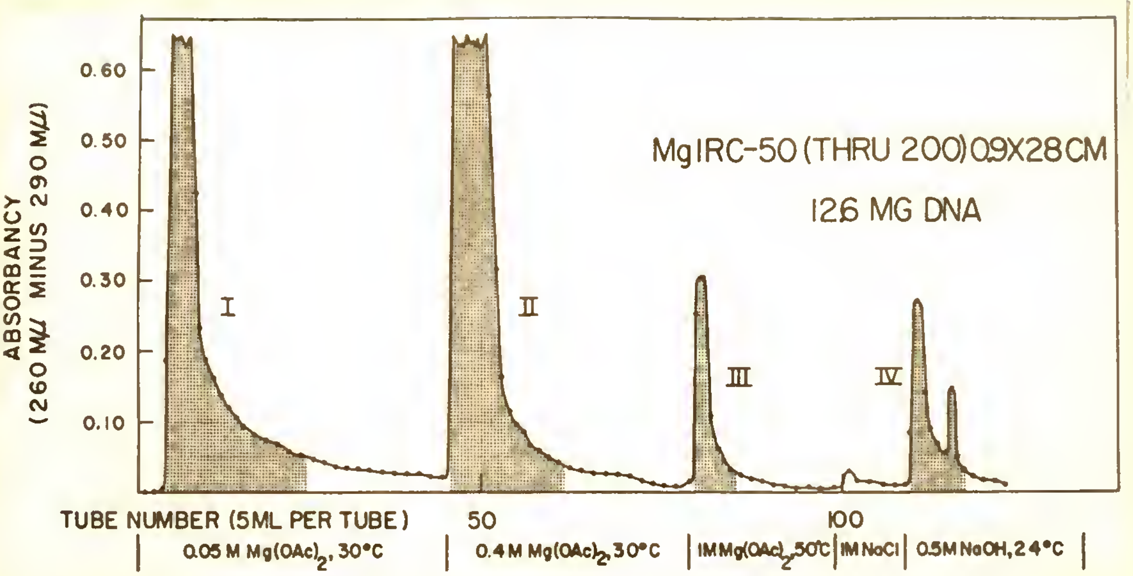

Fig. 13. Stepwise elution from Mg IRC-50 of deoxyribonucleate from Ehrlich ascites tumor cells (EA #9).

TABLE XIII

Five minute in vivo incorporation of C^{14}-formate into the thymine of fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #9)

The fractions were derived from the chromatogram illustrated in Fig. 13.

Fraction	Conditions of elution from Mg IRC-50, 28 x 0.9 cm	Per cent of added sample (13 mg) recovered in fraction	Specific activity of thymine, CPM/μmole
I	0.05 M $Mg(OAc)_2$, 30°	45	429
II	0.4 M $Mg(OAc)_2$, 30°	47	220
III	1 M $Mg(OAc)_2$, 50°	2	1,360
IV	0.5 M NaOH, 24°	3	1,600
Unfractionated, total deoxyribonucleate		(100)	396 ± 7[a]

[a] The variation between duplicate determinations.

Incorporation of H^3-thymidine into fractions of deoxyribonucleate

In vivo study of brief duration -- The possibility that the differential labeling of the thymine discussed above results from a methyl group exchange involving intact deoxyribonucleate molecules was excluded by an experiment in which the incorporation of H^3-thymidine was studied. Cells were removed from each of seven mice five minutes after the injection of 10 μcuries of H^3-thymidine having a specific activity of 360 μcuries per μmole. A 13.1 mg sample of the deoxyribonucleate isolated from the cells (EA #16) was chromatographed on a 28 x 0.9 cm column of Mg IRC-50 as in the previous experiment, except that 1 M NaCl at 50^o, rather than 1 M $Mg(OAc)_2$, was used to elute Fraction III. This modification minimized the precipitation of $Mg(OH)_2$ when 0.5 M NaOH was passed through the column. Because of its asymmetry, Fraction IV was recovered in two portions, IVa and IVb. Thymine was isolated from hydrolysates of the five fractions by paper chromatography and found to have the specific activities given in Table XIV. Although an entirely different precursor was used, the extent of incorporation into chromatographic fractions varied in the same manner as was found originally with C^{14}-formate. That is, the specific activity of thymine in the fraction eluted by 0.4 M $Mg(OAc)_2$ was less than that found in the fractions eluted by 0.05 M $Mg(OAc)_2$ or by the more vigorous conditions.

These data suggest that the observed heterogeneity of incorporation occurs at points which lie beyond the completion of the nitrogenous base in the sequence of reactions leading to the synthesis of deoxyribonucleate. The evidence does not exclude the possibility of an

TABLE XIV

Five minute in vivo incorporation of tritiated thymidine into fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #16)

Fraction	Conditions of elution from Mg IRC-50, 28 x 0.9 cm	Per cent of added sample (13 mg) recovered in fraction	Specific activity of thymine, CPM/mμmole
I	0.05 M $Mg(OAc)_2$, 30°	50	252 ± 3^a
II	0.4 M $Mg(OAc)_2$, 30°	43	132 ± 1^a
III	1 M NaCl, 50°	3	1,040
IVa	0.5 M NaOH, 24°	< 3	623
IVb	0.5 M NaOH, 24°	<3	299
Unfractionated, total deoxyribonucleate		(100)	266 ± 9^a

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[a] The variation between duplicate determinations.

exchange of thymine residues with otherwise intact molecules. However, as was shown in Table XII, the incorporation of C^{14}-formate into adenine and guanine paralleled the incorporation into thymine. This suggests that if exchange occurs, it would involve all bases, and would therefore be tantamount to overall synthesis. It is likely, therefore, that the observed heterogeneity of incorporation of radioactivity is a property associated with the biosynthesis of whole molecules of deoxyribonucleate.

In vivo study of long duration -- As discussed above, differential labeling among chromatographically purified fractions of deoxyribonucleate was reproducibly observed when tumor cells were harvested five to seven minutes after the injection of C^{14}-formate or H^3-thymidine. The specific activities of thymine in fractions of deoxyribonucleate from tumor cells harvested from each of five mice (EA #17) twenty-three hours after injection with 10 μcuries of the H^3-thymidine are given in Table XV. The specific activity of the total deoxyribonucleate was only about 2.7 times that found five minutes after the injection of H^3-thymidine (EA #16, Table XIV). Since the generation time of the tumor cells is eighteen hours, the deoxyribonucleate isolated twenty-three hours after injection would be expected to have a specific activity about one-half as great as that isolated five hours after injection. If it is assumed that the specific activity was close to maximal five hours after injection, then the amount of radioactivity that is incorporated into deoxyribonucleate during the first five minutes after injection is about one-fifth of the maximum amount that can be incorporated under these experimental conditions. Evidently,

TABLE XV

Twenty-three hour in vivo incorporation of tritiated thymidine into fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #17)

Fraction	Conditions of elution from Mg IRC-50, 30 x 0.9 cm	Per cent of added sample (11 mg) recovered in fraction	Specific activity of thymine, CPM/mμmole
I	0.05 M $Mg(OAc)_2$, 30°	22	806 ± 40^a
II	0.4 M $Mg(OAc)_2$, 30°	50	768 ± 59^a
III	1 M NaCl, 50°	3	837
IV	0.5 M NaOH, 24°	< 3	788
Unfractionated, total deoxyribonucleate		(100)	705 ± 31^a

[a] The variation between duplicate determinations.

any thymidine which is not incorporated in deoxyribonucleate shortly after injection rapidly becomes unavailable for incorporation.

After H^3-thymidine is injected, it is possible that the specific activities of pools of precursors at and above the level of thymidine fall almost as abruptly as they rise after the injection of C^{14}-formate (see Fig. 12). Nevertheless, the chromatographic fractions provided by deoxyribonucleate isolated twenty-three hours after injection (Table XV) did not show differences in specific activities, in contrast to the fractions provided by specimens which were isolated five minutes after injection (Table XIV). As studied under these conditions, therefore, the differential labeling of chromatographic fractions is a transient phenomenon. Thus, there is no evidence that molecules in fractions having a high initial specific activity gradually undergo discrete changes in chromatographic behavior. Instead, the specific activity of all fractions approaches that of the total deoxyribonucleate. That is, the specific activities of the fractions initially less radioactive increase at the expense of the "heat peak" fraction, the specific activity of which, however, does not fall below that of the other fractions. The comparatively low specific activity given in Table XV for the thymine of EA #17 total deoxyribonucleate would indicate that the average specific activity of thymine in the material that remained on the column was about 500 CPM/$m\mu$mole.

In vitro study of brief duration -- As discussed in the preceding section, about one-fifth of the maximum possible incorporation of thymidine occurs during the first five minutes after injection. In an effort to reduce the extent of incorporation still further, an *in vitro* experiment was performed. Cells were removed from the peritoneal cavities of six mice with the aid of about 2 ml of saline per mouse, and transferred to a glass vial at 35°-37° C, a process which required about sixty minutes. After adding 8 μcuries of the tritiated thymidine, the cell suspension was incubated for two minutes with occasional stirring. The deoxyribonucleate (EA #15) was isolated and chromatographed to provide the data which are given in Table XVI. Although the total amount of precursor incorporated was small, the distribution of specific activities is similar to that seen previously in the five minute *in vivo* experiments. It is difficult to compare the extent of incorporation with previous experiments since an equivalent of fewer μcuries per mouse was provided in this *in vitro* experiment. In view of the loss of the capacity for synthesis of deoxyribonucleate by cells maintained under *in vitro* conditions, it is possible that incorporation occurred only in the last cells transferred to the incubation vessel. Nevertheless, preferential incorporation of thymidine into "heat peak" material was even more pronounced in this two minute *in vitro* experiment than in the five minute *in vivo* experiments described above. It will be of interest to study even shorter intervals of incorporation.

TABLE XVI

Two minute in vitro incorporation of tritiated thymidine into fractions of deoxyribonucleate from Ehrlich ascites tumor cells (EA #15)

Fraction	Conditions of elution from Mg IRC-50, 30 x 0.9 cm	Per cent of added sample (11 mg) recovered in fraction	Specific activity of thymine, CPM/mμmole
I	0.05 M $Mg(OAc)_2$, 30°	33	2.75 ± 0.25[a]
II	0.4 M $Mg(OAc)_2$, 30°	44	1.75 ± 0.05[a]
III	1 M NaCl, 50°	2	16.00
IV	0.5 M NaOH, 24°	< 2	12.00
Unfractionated, total deoxyribonucleate		(100)	2.95 ± 0.05[a]

[a] The variation between duplicate determinations.

CRITICISM OF THE CHROMATOGRAPHIC STUDIES

The results of the chemical and biological studies presented in the preceding sections emphasize the need for further information regarding the molecular parameters which govern the specific interaction of deoxyribonucleate with Mg IRC-50. The chemical studies indicate that under certain conditions Mg IRC-50 has an affinity for all molecules of deoxyribonucleate, but that in general the affinity of the resin for molecules rich in adenine and thymine is stronger than for molecules rich in guanine and cytosine. Accordingly, the rank in the hierarchy of affinity of molecules of deoxyribonucleate for Mg IRC-50 appears to be governed to some extent by total base composition. The possibility that relatively minor chemical factors, in addition to total base composition, may modify this affinity is illustrated by a consideration of the distribution of 5-methylcytosine among the successive chromatographic fractions. While the mole per cent of cytosine of extreme fractions changes by factors of 1.3 and 1.2 in the specimens from calf thymus and Ehrlich ascites tumor cells, respectively, the mole per cent of 5-methylcytosine changes by factors of 5.2 and 2.0. In time, detailed structural studies may reveal whether the affinity of deoxyribonucleate for Mg IRC-50 is lessened by the relative preponderance of the satellite base per se, or whether this affinity depends on exaggerated base sequences which are correlated with enrichment by 5-methylcytosine. During the course of this work, improved chromatographic procedures were developed for the analysis of pyrimidine nucleoside 3',5'-diphosphates, dinucleoside triphosphates, and higher homologues present in acid (39) and diphenylamine (40) hydrolysates of deoxyribonucleate. By providing evidence with regard to the relative clustering of purines and pyrimidines, these techniques may lead to a clearer definition of distinctive features of the structure of deoxyribonucleate to which the interaction with Mg IRC-50 is sensitive. When more discriminating criteria for the characterization of deoxyribonucleates are applied, the differences between the chromatographic fractions may prove to be more profound than presently suspected. That is, "the differentiation between nucleic acids on the basis of differences in the contents of their several nitrogenous constituents [has been] a comparatively crude expedient, since it does not permit a distinction between sequence variations that are not accompanied by a change in total composition" (5). The extent to which particle weight influences the chromatographic behavior of molecules of deoxyribonucleate on columns of Mg IRC-50 has not as yet been determined. It would be of interest to apply techniques such as have been used by Schumaker and Schachman (11) in order to determine the distribution of sedimentation coefficients within the chromatographic fractions. As was noted in the Introduction, the chromatographic procedures of Bendich and co-workers provide molecules having progressively increasing sedimentation coefficients but essentially unchanging base composition (9). The last fractions eluted from columns of ECTEOLA-cellulose have an average sedimentation coefficient higher than that of the total, unfractionated material. However, the successive fractions also exhibit a progressively greater heterogeneity with respect to distribution of sedimentation coefficients. For this reason it is difficult to compare fractions obtained by use of Mg IRC-50 with fractions obtained using columns of ECTEOLA-cellulose. Different principles appear to underlie the two fractionation methods. In contrast to the results obtained by Bendich and co-workers, the procedures of Chargaff (4), of Butler (6), and of Brown (8) and their co-workers provide fractions of deoxyribonucleate which exhibit the same trends in base composition as the fractions eluted from columns of Mg IRC-50. The use of Mg IRC-50 for the isolation of such fractions of deoxyribonucleate presents a number of advantages not offered by the previous procedures. There is little doubt about the reproducibility of the Mg IRC-50 chromatograms. The resin is readily available commercially and several lots have provided essentially identical results. The earlier procedures are difficult to standardize and to reproduce because they depend on the use of histones of uncertain homogeneity. Moreover, "denatured" histone and undenatured histone yield considerably different chromatographic results (8). Although the histone-chloroform gel procedure causes serious losses of transforming activity,[16] passage through columns of Mg IRC-50 or through certain histone-cellulose columns (8) has been found to be innocuous in this respect.

The chromatographic studies have not as yet been concerned directly with the problem of whether each specimen of deoxyribonucleate consists of a spectrum of differently constituted molecules or rather, consists of a mixture of a relatively few different molecules. The use of Mg IRC-50 by itself or in conjunction with alternative fractionation methods may eventually provide information pertinent to this problem. In this regard, determination of the distribution of sedimentation coefficients (10, 11) as well as density gradient analysis in CsCl solutions (12) and studies of the temperature dependence of ultraviolet absorbancy (13) may prove to be convenient techniques for evaluating the extent of heterogeneity in fractions obtained from columns of Mg IRC-50. The interpretation of the chromatographic studies would also be aided by definitive knowledge of the chromatographic behavior of specimens of deoxyribonucleate which are, in effect, initially more homogeneous than those examined thus far. Such specimens could be obtained by proper choice of biological origin, by the preliminary use of alternative fractionation techniques, or by the application of conditions, such as controlled heating, which preferentially destroy the native structure of certain molecules without altering others.

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[16] C. F. Crampton, E. Chargaff, and R. D. Hotchkiss, unpublished observations (1953).

The evaluation of molecular parameters which underlie the chromatographic fractionations in the biological studies is even more complicated. Unlike the "crude expedient" of measurements of total base composition, measurements of transforming activity need not be the result of a positive contribution by all of the molecules present in a given fraction. It will be recalled that the transforming activity of specimens of deoxyribonucleate from _Pneumococcus_ was distributed throughout most of the chromatographic fractions. This could result either from the existence of the same biological activity in molecules of different composition or from the association of all of the biological activity with molecules of discrete composition which, for some reason, interacted with Mg IRC-50 to an equivalent degree over a diffuse range of concentrations of $Mg(OAc)_2$. Similar uncertainties pertain to the results of the incorporation experiments in which the specific activities of thymine changed considerably throughout the successive fractions. An incorporation period of five minutes corresponds to 1% of the average time of 8.4 hours which each cell devotes to the synthesis of deoxyribonucleate during the growth cycle (17). Since the generation time of the cells is about eighteen hours (17), roughly half of all the cells present in an exponentially growing culture are engaged in synthesizing deoxyribonucleate. If utilization of the precursors began immediately after injection in all cells which were synthesizing deoxyribonucleate, the isolated specimens would be about "0.5% labeled." However, it is not known whether many molecules are slightly labeled, or a small number of molecules are intensively labeled. Moreover, the occurrence of radioactivity in all chromatographic fractions poses the same problem noted above with respect to the ubiquity of transforming activity among the fractions from pneumococcal specimens.

The characterization of fractions by measurements of activities is hazardous, since the distribution of activity may be governed by minor structural peculiarities not shared by all of the molecules present in the fractions. That is, incipiently labeled deoxyribonucleate might be expected to behave abnormally upon chromatography because a double-stranded structure had not been completed when the cells were chilled prior to isolation of the specimen. Moreover, the chromatographic behavior of the small absolute quantities of labeled deoxyribonucleate could also be dictated chiefly by associated contaminants, such as protein, polysaccharide, or lipid, which had been attached during the process of replication. In this connection, it will be recalled that the specimen from rat liver contained a large amount of protein but gave chromatographic patterns similar to those provided by calf thymus. On the other hand, the preparations from Pneumococcus, which also contained large amounts of protein (and ribonucleate), gave quite different patterns. A systematic study of the protein content of chromatographic fractions was not attempted routinely because of the presence of only trace amounts of protein in most of the total specimens.

The fact that columns of Mg IRC-50 are able to separate different molecules of deoxyribonucleate rests not upon the biological studies but rather upon the chemical studies of composition and upon the results of the rechromatography experiments. Nevertheless, the biological studies have shown that, under certain experimental conditions, affinity for Mg IRC-50 is in some way related to the metabolic origin of molecules of deoxyribonucleate.

POSSIBLE SIGNIFICANCE OF THE BIOLOGICAL STUDIES

The results of the incorporation experiments are summarized in Table XVII, which lists the relative specific activities of thymine in the two chromatographic fractions between which the greatest differences were usually obtained. As can be seen, preferential labeling of chromatographically different fractions of deoxyribonucleate from Ehrlich ascites tumor cells was observed following brief incorporation periods. The data again direct attention to the "heat peak" fraction, the unusual properties of which were discussed in earlier sections. It will be recalled that material in this position may be partly artifact and partly an authentic component present in the deoxyribonucleate submitted to fractionation. The base composition of the material resembles that of total deoxyribonucleate and therefore is not in line with the progressive increase in the ratio, (adenine + thymine)/(guanine + cytosine), otherwise noted throughout the chromatogram. Of particular significance is the fact that the specimens of deoxyribonucleate from Pneumococcus provided relatively large amounts of "heat peak" material in which specific transforming activity was comparable to that of the total specimen.

Had the incorporation experiments compared in Table XVII dealt with synchronously growing cells, the preferential labeling could be explained on the premise that chromatographically different molecules are synthesized sequentially. Radioautographic studies following

TABLE XVII

Relative specific activity of thymine in chromatographic fractions of deoxyribonucleate from Ehrlich ascites tumor cells

The specific activities are relative to that of thymine in the respective unfractionated specimens of total deoxyribonucleate. The figures are based on results presented in greater detail in the tables indicated.

Table	Specimen	Mode of labeling		Relative specific activity of thymine in chromatographic fractions	
		Precursor	Conditions of incubation	Fraction eluted by 0.4 M $Mg(OAc)_2$	"Heat peak"
XI	EA #7	C^{14}-formate	5 min, *in vivo*	(0.51)	3.2
XIII	EA #9	C^{14}-formate	5 min, *in vivo*	0.56	3.4
XIV	EA #16	H^3-thymidine	5 min, *in vivo*	0.50	3.9
XV	EA #17	H^3-thymidine	23 hr, *in vivo*	1.1	1.2
XVI	EA #15	H^3-thymidine	2 min, *in vitro*	0.59	5.4

administration of H^3-thymidine suggest that sequential synthesis of deoxyribonucleate actually occurs in chromosomes of grasshopper testes (41) as well as in plant tissues (42). However, in the present studies, ascites tumor cells at all stages of replication were examined, thereby excluding specific temporal sequence as a possible explanation of preferential labeling.

The synthesis of deoxyribonucleate by Ehrlich ascites tumor cells occurs during 8.4 hours of the interphase period of the life cycle of these cells (17). It is possible that the biosynthesis of individual molecules of deoxyribonucleate requires a large portion of this time. If so, radioactivity would be confined to limited regions of large numbers of incomplete molecules after brief incorporation periods. Accordingly, the observed chromatographic distribution of radioactivity could depend largely upon heterogeneity with respect to degree of completion.

On the other hand, the complete synthesis of molecules of deoxyribonucleate may require only a few seconds.[17] That is, the 8.4 hour period of synthesis might encompass the successive syntheses of many different molecules which are started and finished at different times in individual cells. In this case different molecules could attain different specific activities if there were a more rapid incorporation of the labeled precursor into one group of molecules than into another. Such preferential incorporation could occur in at least three ways.

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[17] It is also possible that some molecules of deoxyribonucleate are synthesized more rapidly than others because of restrictions imposed by the statistical ease with which available precursors can be aligned and polymerized in order to complete characteristic sequences of certain of the molecules. Moreover, molecules containing large amounts of 5-methylcytosine might be assembled less rapidly than molecules which do not contain this satellite base.

1) The specimens of deoxyribonucleate may contain a small group of macromolecular precursors from which all molecules of deoxyribonucleate are derived. Such precursors would exhibit a more rapid increase in specific activity than would the product molecules after the injection of isotopic compounds. Moreover, such precursor molecules would subsequently lose their activity more rapidly than would the product molecules. It is possible that deoxyribonucleate in the "heat peak" corresponds to such precursor material. The "heat peak" fraction differs from the bulk of the nucleate not only by its very high rate of incorporation of radioactive precursors, but also by its abnormal affinity for Mg IRC-50. Moreover, the composition of "heat peak" deoxyribonucleate resembles that of the whole specimen, a property that would be expected for a fraction from which all the molecules of a preparation arise. Precursor, or incomplete deoxyribonucleate, might well be expected to differ structurally from completed nucleate in a manner which could cause a greater affinity for the resin. Precursor deoxyribonucleate could be single-stranded (43), multi-stranded (44,45), or associated with materials which are not attached to completed molecules. On the other hand, the specific activity of precursor deoxyribonucleate should approach zero a long time after administration of isotope, a requirement which is not met by the "heat peak" material. The specific activity of all fractions, including "heat peak," approached a similar value twenty-three hours after injection. It is

quite possible, however, that precursor deoxyribonucleate in "heat peak" is contaminated by a large amount of ordinary nucleate. In such an event, the large amount of radioactive product deoxyribonucleate which is eluted non-specifically in "heat peak" would obscure the low specific activity of the precursor material. The differences in the specific activities of the chromatographic fractions other than "heat peak" could be attributable to a form of precursor deoxyribonucleate which emerges together with the early chromatographic fractions from Mg IRC-50.

2) If the different molecules of deoxyribonucleate in a single cell are synthesized from independent pools of low molecular weight precursors they would become labeled at unequal rates if the pools differed in size. Those molecules formed from the smallest pools of precursors would incorporate the added label most rapidly.

3) If molecules of deoxyribonucleate differed with respect to the rate at which they were synthesized from a single pool of precursors, differences in specific activity could result if there were rapid turnover in a portion of the molecules, or possibly, if different molecules were synthesized at specifically related times not related to their chromatographic behavior.

The experimental observation of preferential labeling of different fractions of deoxyribonucleate is not without precedent in the literature. For example, Bendich, Russel, and Brown (46) found that deoxyribonucleate extracted from rat tissues with 10% NaCl at 85° C yielded fractions which differed in their solubility in saline. The two fractions of deoxyribonucleate isolated from various organs several days after injection of C^{14}-formate contained different amounts of radioactivity. However, the specific activities of different bases from the fractions of a single organ did not parallel one another, nor did different organs consistently show a greater incorporation into one of the fractions than into the other. The authors conclude, "the results not only show that [deoxyribonucleate] of any single organ is not metabolically homogeneous, but that the individual bases of the nucleic acids of a given organ are renewed at dissimilar rates, and that the pattern of renewal varies from organ to organ." However, the differences reported for fractions from a single organ were smallest with respect to thymine, the base least likely to be contaminated by material derived from highly radioactive ribonucleate. The greatest difference in specific activity of thymine occurred in a specimen from small intestine, where specific activities of 614 CPM/μmole and 779 CPM/μmole were reported for Fractions 1 and 2, respectively. In contrast to the experiments of Bendich et al., the studies summarized in Table XVII have revealed large specific activity differences among fractions with respect to a base which is not present in ribonucleate from most sources. As has been noted previously (Table XII), parallel results were obtained with one series of fractions by specific activity measurements of guanine, which is present in both ribo- and deoxyribonucleates. In addition, deoxyribonucleate was isolated by use of the urea medium which has been found to provide specimens free from appreciable amounts of uracil, which is a characteristic constituent of ribonucleate. Finally, extremely similar patterns of incorporation were given both by H^3-thymidine and C^{14}-formate, precursors which are utilized for the synthesis of the two types of nucleate with considerably different efficiencies.

A second report of metabolic heterogeneity of deoxyribonucleate is that of Friedkin and Wood (47), who incubated chicken bone marrow cells *in vitro* for two hours with C^{14}-thymidine. The incubation mixtures were then frozen, lyophilized, and extracted with organic solvents. After stirring in 0.9% NaCl, the material was treated with sodium dodecyl sulfate by the procedure of Kay *et al*. (21). Three successive extractions provided three fractions of deoxyribonucleate. The specific activity of the thymidine isolated from enzymatic hydrolysates varied by as much as 3.3 from one fraction to another. The interesting results of Friedkin and Wood merit further study. They are, however, somewhat difficult to interpret since the fractions were obtained from unpurified deoxyribonucleate by ill-defined techniques not known to give fractions which differ in other respects. Moreover, the use of a system consisting of an initially heterogeneous population of cells, in which there is not known to be a net synthesis of deoxyribonucleate, invites uncertainties which were avoided as far as possible in the present work.

SUMMARY

Variables affecting the specific interaction between Mg IRC-50 and highly purified specimens of deoxyribonucleate were examined. Conditions optimal for the analysis of molecules of different chemical composition were developed and applied to specimens of deoxyribonucleate isolated from Ehrlich ascites tumor cells which had incorporated C^{14}-formate or H^{3}-thymidine for very brief intervals of time. Large differences between the fractions with respect to the specific activity of their thymine residues were found, suggesting that the columns of Mg IRC-50 may be used to separate molecules of different biological origin as well as of different chemical composition. The basis of the interaction of Mg IRC-50 and deoxyribonucleate is discussed critically and explanations are offered for the preferential incorporation of precursors into chromatographically different fractions.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Medicine and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 13, 1960

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